ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 43

★

REDACTORES

Y. REENPÄÄ *Helsinki* — *F. BUCHTHAL* *København* — *R. NICOLAYSEN* *Oslo*

Y. ZOTTERMAN *Stockholm* — *U. S. VON EULER (EDITOR)* *Stockholm*

COLLABORANTES

G. AHLGREN (Lund), E. L. BACKMAN (Uppsala), E. BÁRÁNY (Uppsala), S. BERGSTRÖM (Stockholm), C. G. BERNHARD (Stockholm), G. BLIX (Uppsala), A. CARLSTEN (Göteborg), R. EGE (København), H. v. EULER (Stockholm), A. FÖLLING (Oslo), B. GERNANDT (Göteborg), L. GOLDBERG (Stockholm), R. GRANIT (Stockholm), E. HAMMARSTEN (Stockholm), E. HANSEN (København), K. HARTIALA (Åbo), E. HOHWÜ-CHRISTENSEN (Stockholm), I. HOLM-JENSEN (Århus), E. JALAVISTO (Helsinki), E. JORPES (Stockholm), F. LEEGAARD (Oslo), J. LEHMANN (Göteborg), G. LILJESTRAND (Stockholm), E. LUNDSGAARD (København), O. MELLANDER (Göteborg), J. MOLLAND (Oslo), K. MØLLER (København), S. ØRSKOV (Århus), P. B. REHBERG (København), A. V. SAHLSTEDT (Stockholm), C. SCHMITERLÖW (Stockholm), F. SCHÖNHEYDER (Århus), P. E. SIMOLA (Helsinki), K. SJÖBERG (Stockholm), T. SJÖSTRAND (Stockholm), T. TEORELL (Uppsala), H. THEORELL (Stockholm), H. USSING (København), B. UVNÄS (Stockholm), O. WALAAS (Oslo), A. V. VARTIAINEN (Helsinki), A. WESTERLUND (Uppsala), A. I. VIRTANEN (Helsinki), G. ÅGREN (Uppsala)

STOCKHOLM 1958 · KUNGL. BOKTRYCKERIET P. A. NORSTEDT & SÖNER

Acta physiol. scand. 1958. 43. 1—7.

From the Department of Physiological Chemistry, University of Lund, Lund, Sweden.

On the Regulation of Bile Acid Formation in the Rat Liver.

By

S. BERGSTRÖM and H. DANIELSSON.

Received 20 February 1958.

It has recently been shown at this Institute that the half-life of bile acid molecules in a normal 200 g rat was about 2.5 days. As the total amount of bile acids present in the enterohepatic circulation appears to be about 25—30 mg, the daily synthesis is thus about 5—6 mg per day (LINDSTEDT and NORMAN 1956, BERGSTRÖM and ERIKSSON).

In a bile fistula rat, however, the daily excretion of bile acids in the fistula bile is considerably greater as was first found by THOMPSON and VARS (1953) who analyzed only the cholic acid content of the bile. ERIKSSON (1957) has recently made a detailed study of the bile acid excretion in fistula rats, analyzing both the cholic and chenodesoxycholic acid present. After excretion of the bile acids present in the enterohepatic circulation (6—8 hours), the excretion rapidly increased to 50—70 mg per day, a rate that was reached already the second or third day and then remained at this level.

The object of this work was to make a preliminary study of the factors involved in the regulation of the bile acid synthesis in the normal rat. The most obvious factor involved is the enterohepatic circulation of the bile salts. In this way the liver is continuously supplied with bile salts, absorbed from the intestine via the portal route and it is known that they are very efficiently

removed by the liver cells. It seemed likely that the formation of bile acids in the liver cells in some way is influenced by the amount of bile acids or bile salts supplied to the liver through the portal blood. In bile fistula animals that have a bile acid synthesis 10—20 times the normal, no bile acids reach the liver, as the bile is directly excreted via the fistula outside the body and no bile acids reach the intestine.

In the intact rat the concentration of bile salts in the portal blood depends mainly on the number of times that the bile salt pool circulates from the liver to the intestine and back to the liver. In this connection the distribution of the bile acids in the steady state also might be of interest. After administration of tracer doses of labelled bile acids, NORMAN and SJÖVALL (1958) have studied the distribution after different times. They have found that about 85 per cent was present in the intestinal content, 10—12 per cent in the intestinal wall and only the remaining few per cent in the liver. It might be mentioned that the rat does not have a gall-bladder.

An indication of the rate of the enterohepatic circulation under normal conditions can be gained from ERIKSSON's determinations of the bile salt excretion in the fistula during the first hours following the operation, when he found values corresponding to 2—5 mg per hour. Even if the anesthesia and the trauma of the operation might have had a considerable influence, these data must presumably be considered as minimal values due to the influence of intestinal mobility etc. Whatever the importance that can be attached to quantitative data obtained under these conditions, it is obvious that the enterohepatic circulation is a relatively rapid process that can recirculate the bile salts present many times per day in the normal rat.

In order to simulate normal conditions in a bile fistula rat as far as the amount of bile salts secreted into the intestine via the bile duct was concerned, the following experiments were done.

After a plastic cannula had been tied into the upper part of the cut bile duct, as in the usual bile fistula operation, a similar cannula was also tied into the lower part. The latter was connected with an arrangement for continuous injection of half a milliliter per hour. Usually saline was injected in which the desired amount of the sodium salt of the bile acid under investigation had been dissolved.

As the amount of cholic acid normally formed in the liver is small compared to the amount that might have to be injected, the bile

acid injected had to fulfill the following requirements: the salt should be clearly separable from cholic acid and not form cholic acid or other metabolites that might simulate cholic acid.

One such acid is chenodesoxycholic acid which, as has been found by BERGSTRÖM and SJÖVALL (1954), only to a minor extent is hydroxylated during its passage through the liver. Furthermore, the products formed can be separated from cholic acid by chromatography thus making possible the determination of the cholic acid synthesized. The latter has been determined either as free acid by reversed-phase column chromatography or as tauroconjugate by quantitative paper chromatography (SJÖVALL 1955 a), as the chenodesoxycholate-metabolites show no U. V. absorption under the conditions that taurocholate is measured.

We have now determined the production of cholic acid in bile fistula rats of different weights, that were continuously injected with 2.5—15 mg of sodium taurochenodesoxycholate (NaTCD) per hour in the way just described.

Experimental.

In the three series of experiments white male rats of the Institution stock weighing about 200, 250 and 300 g respectively were used. Under ether anesthesia the bile duct was ligated approximately in the middle of its course, and one plastic cannula was inserted in the upper part of the bile duct in order to collect the bile from the liver and another into the lower part of the duct. This latter cannula was connected to an apparatus enabling continuous injection of 0.5 ml of fluid per hour. The NaTCD was dissolved in an appropriate volume of saline and the pH of the solution adjusted to 7.2. The bile was collected in 95 % ethanol in 24 hour portions. During the experiment the rat had access to white bread and water.

The amount of cholic acid in the bile was determined either by paper chromatography as the tauro-conjugate, when also tauro-chenodesoxycholic acid could be assayed, or by reversed-phase column chromatography as free acid.

In the former case the bile was diluted to 50 ml with ethanol and 25—50 μl were put on Whatman 3 filter paper and chromatographed according to SJÖVALL (1955 b) using isoamyl-acetate: heptane 85:15 as mobile and 70 per cent aqueous formic acid as stationary phase. After 1/2 hour saturation of the paper it was run ascending for 16 hours. The acids were detected by spraying with 10 per cent phosphomolybdic acid in ethanol, eluted from the paper with ethanol and measured as their sulfuric acid chromogens, as described by SJÖVALL (1955 a) and ERIKSSON and SJÖVALL (1955).

In the latter case the bile was hydrolyzed with N NaOH in 50 per cent ethanol in a sealed steel tube at 110°C for 6 hours. After dilution with water and acidification with hydrocholic acid, the bile acids were extracted three times with ether. The combined ether-extracts were washed with water until neutral and then evaporated to dryness in vacuo. The residue was chromatographed on hydrophobic kieselguhr with phase system C (NORMAN 1953). The effluent was titrated with 0.02 N sodium hydroxide in methanol. The amount of cholic acid was calculated from the titration values.

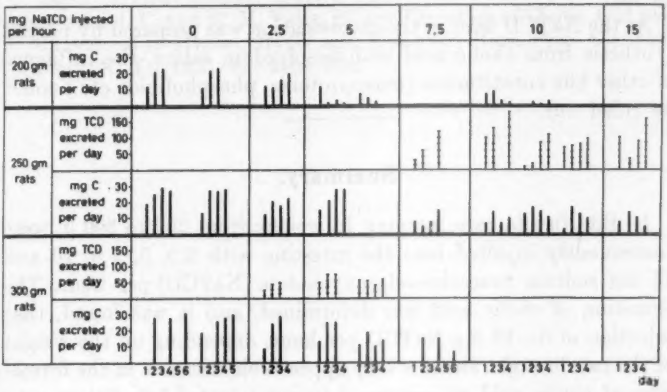
Results.

Table I shows the effect of continuous injection of NaTCD in different concentrations on the excretion of cholic acid in bile fistulas on rats weighing approx. 200, 250 and 300 g respectively. It can be seen that an amount of 5 mg or more of NaTCD per hour to a 200, 7.5 mg or more to a 250 and 300 g rat brings about an appreciable decrease as compared to animals injected with saline in the cholic acid synthesis. The effect is noticed from the second day on, when the cholic acid present in the enterohepatic circulation has been excreted. The rats injected with 0.9 per cent NaCl show the normal pattern of bile acid excretion in bile fistula rats, where a maximum is reached on the second or third day and then maintained at this level, which corresponds to a daily excretion of 25—30 mg of cholic acid, corresponding to a synthesis 10—20 times higher than that of the intact animal.

The "threshold" concentration of NaTCD giving a depression of cholic acid formation being established for each group of animals, it is found that the size of depression with this "threshold" concentration and the possibility to increase it by injecting higher concentrations of NaTCD varies considerably. In the group with 200 g rats 5 mg or more of NaTCD per hour suppress the cholic acid synthesis to such an extent that the amount of cholic acid excreted is of the same order of magnitude as the formation of this acid in the intact rat. In the other two series of rats, those weighing 250 and 300 g, the effect is not dramatic as in the first group. 7.5 mg of NaTCD per hour to the 250 g rats, with which the greatest number of experiments were done, cause a considerable decrease in the cholic acid excretion. Injection of 10 or 15 mg per hour gives variable results, but when the amount of NaTCD absorbed, measured as amount excreted in the bile fistula, is taken into consideration, there is a fairly good correlation between amount of NaTCD absorbed and the magnitude of suppression of cholic

Table I.

Excretion of cholic acid, measured as free acid and as tauro-conjugate, in bile fistulas on rats of different weights, that have been continuously injected with different concentrations of sodium taurochenodesoxycholate. NaTCD = sodium taurochenodesoxycholate. C = cholic acid. TCD = taurocholic acid.



acid formation. It is thus found that when 75—100 mg of NaTCD per day are absorbed, the effect on cholic acid synthesis sets in, the further depression of which then will depend on how much more NaTCD is absorbed.

Discussion.

The results obtained indicate strongly that the formation of bile acids in the liver is regulated by the amount of bile acids supplied to the liver via the portal blood. The concentration of bile acids in the portal blood under normal conditions is not known, nor is the number of times per day that the bile salt pool circulates. Some tentative calculations can, however, be made based on the results of this investigation. It was found that 5 mg of NaTCD per hour to a 200 g bile fistula rat resulted in an inhibition of cholic acid synthesis to the extent that the amounts formed correspond to those calculated for the intact animal. This would then mean that the liver has to be supplied with about 5 mg of bile acid per hour in order not to synthesize more than what covers the daily loss of bile acids under normal conditions, *i. e.* 4—5 mg.

The concentration of bile acids in the portal system would then be in the neighbourhood of only one mg per 100 ml blood, assuming a portal blood flow of around 10 ml per minute. Further, the number of times per day that the enterohepatic bile salt pool circulates would thus be approx. 6 times, if it amounts to approximately 20 mg.

As the NaTCD used in the investigation was prepared by partial synthesis from cholic acid and dissolved in saline, the influence of other bile constituents (mucoproteins, phospholipids etc.) could be ruled out.

Summary.

1. Bile fistula rats, ranging in weight from 200 to 300 g, were continuously injected into the intestine with 2.5, 5, 7.5, 10 and 15 mg sodium taurochenodesoxycholate (NaTCD) per hour. The excretion of cholic acid was determined, and it was found, that injection of 5—10 mg NaTCD per hour, depending on the weight of the rat, brought about a very appreciable decrease in the formation of cholic acid as compared to non-injected bile fistula rats. In the case of rats weighing approx. 200 g, the values found are of the same order of magnitude as is calculated for the synthesis of cholic acid in the intact rat.

2. It is concluded, that the concentration of bile acids supplied to the liver via the portal blood, influences the rate of synthesis of bile acids in the liver.

This investigation was supported by grants from Statens Medicinska Forskningsråd and Knut och Alice Wallenbergs Stiftelse.

The technical assistance of Miss K. Hyllén and Miss B. Jönsson is gratefully acknowledged.

References.

BERGSTRÖM, S. and S. ERIKSSON, To be published.

BERGSTRÖM, S. and J. SJÖVALL, Occurrence and Metabolism of Chenodesoxycholic Acid in the Rat. Acta chem. scand. 1954. *8*. 611—616.

ERIKSSON, S., Biliary Excretion of Bile Acids and Cholesterol in Bile Fistula Rats. Proc. Soc. exp. Biol. (N. Y.) 1957. *94*. 578—582.

ERIKSSON, S. and J. SJÖVALL, The Absorption Spectra of Conjugated Bile Acids in Sulfuric Acid. Arkiv f. Kemi *8*. 1955. 311—315.

LINDSTEDT, S. and A. NORMAN, The Turnover of Bile Acids in the Rat. Acta physiol. scand. 1956. *38*. 121—128.

NORMAN, A., Separation of Conjugated Bile Acids by Partition Chromatography. Acta chem. scand. 1953. *7*. 1413—1419.

NORMAN, A. and J. SJÖVALL, To be published 1958.

SJÖVALL, J., (a) Quantitative Determination of Bile Acids on Paper Chromatograms. Arkiv f. Kemi 1955. *8*. 317—324.

SJÖVALL, J., (b) New Solvents for the Separation of Bile Acids with Paper Chromatography. Arkiv f. Kemi 1955. *8*. 299—301.

THOMPSON, J. C. and H. M. VARS, Biliary Excretion of Cholic Acid and Cholesterol in Hyper-, Hypo- and Euthyroid Rats. Proc. Soc. exp. Biol. (N. Y.) 1953. *83*. 246—248.

Acta physiol. scand. 1958. 43. 8—14.

From the Department of Physiology, Karolinska Institutet, Stockholm 60, and the Department of Pathology I, University of Uppsala, Sweden.

The Influence of Hypoglycemia on the Histologic Structure of the Alpha Cells in the Pancreas.

By

BERNT HÖKFELT and GÖSTA HULTQUIST.

Received February 4 1958.

In 1942, JANBON, LAZERGES and METROPOLITANSKI demonstrated that p-amino-sulfonamide-isopropyl-thiodiazole reduces blood sugar in man. Several other related substances were subsequently found to have the same effect (LOUBATIÈRES 1944, 1946, BOVET and DUBOST 1944). During the past few years, first sulfanilyl-n-butylurea (BZ 55), and then p-tolyl-sulfonyl-n-butylurea (D 860), both of which have a hypoglycemic effect, have been used, especially by German investigators, in the peroral treatment of certain forms of diabetes.

The mechanism underlying the blood sugar-reducing effect of BZ 55 and D 860 is not entirely clear. A number of investigators have, however, advanced the suggestion that the blood sugar reducing sulfonamides perhaps interfere with the glucagon metabolism in the organism. It has, for instance, been suggested that the substances in question may cause primary damage and degeneration of the alpha cells in the pancreas and in consequence eliminate the glucagon, the disappearance of the latter causing a drop in the blood sugar level (FERNER and RUNGE 1956 a, b, ACHELIS and HARDEBECK 1955, and others). Other authors consider that the alpha cell changes might represent an adaptation to the hypoglycemic state (KRACHT, v. HOLT and v. HOLT 1957).

With a view to ascertaining whether the changes in the cell

islets in the pancreas observed after treatment with BZ 55 are primary, or whether they are secondary to the hypoglycemia produced, we have made a comparative cytologic investigation into the alpha cell structure following hypoglycemia, after the administration of BZ 55 on the one hand and of insulin on the other.

Material and Methods.

All examinations were carried out on white rabbits (2.0—2.5 kg), which had been starved for 15 hours preceding the start of the experiment.

BZ 55 was administered intravenously in a single dose, 0.5 g/kg of body weight, in the form of Na salt. Regular insulin was injected subcutaneously, 0.35 I.U./kg, in repeated doses at intervals of 3 to 4 hours. The blood sugar was determined according to the method of Hagedorn and Jensen, samples being taken at intervals of 30—60 minutes.

The animals were killed by air embolism 4 to 24 hours after the administration of BZ 55 or the first insulin injection. In a few instances, insulin was given once a day (0.35 I.U./kg) over a period of two weeks; the animals were then killed 24 hours after the last insulin dose. The pancreas was fixed in Bouin's fluid. For the sake of comparison, the following stainings were prepared, with the specific purpose of demonstrating the alpha cells, namely, chromhematoxylin-phloxin and azan, both by the Gomori methods, and Gomori's chromhematoxylin-Ponceaufuchsin. Although the beta cells were stained by these methods, they were seen with special intensity when Gomori's paraldehyde-fuchsin stain was used. In some cases glycogen staining according to the Best method was used. The nuclear size was measured by tracing off the nuclei in a magnification of approximately 1,500 and then determining the surface of the sketches in μ^2 with a planimeter.

Results.

It will be seen from Table I that in the first series of insulin experiments the stainability of the alpha cells decreased considerably with all methods used. The decrease was most pronounced after staining with chromhematoxylin-phloxin, while after chromhematoxylin-Ponceaufuchsin it was less marked. In addition, there was remarkable variation in the stainability of the alpha cells, the granulation in some of the cells being scarcely noticeable while in others it was almost normal.

Only a few data from BZ 55-treated animals have been included in Table I. It will be seen that the typical changes in stainability

Table I.

Stainability of alpha cell granulation following hypoglycemia induced by insulin and BZ 55. Stainability has been graded from 1 to 10, the last figure corresponding to stainability in control animals, all figures representing mean stainability as judged from a number of individual alpha cells.

Rabbit no.	Alpha cells			Agent administered	Duration of hypoglycemia hrs.	Lowest blood sugar found; mg%
	Chromhematoxylin-phloxin	Azan	Chromhematoxylin-Ponceaufuchsin			
1	6	9	7	Insulin	4	50
2	2	4	6	»	9	35
3	3	5	7	»	9	38
4	4	5	6	»	9	45
5	1	4	6	»	9	33
6	3	6	6	»	10	42
7	2	7	7	»	11	52
8	0	5	5	»	12	29
9	4	7	10	»	12	43
10	—	—	7	»	12	33
11	2	4	6	»	20	49
12	—	—	6	»	24	41
1	5	6	7	BZ 55	4	67
2	4	6	7	»	10	69
3	2	4	6	»	24	52
4	2	4	7	»	24	42
5	2	7	7	»	24	69

of the alpha cells recorded after insulin treatment were also present after hypoglycemia following BZ administration. After both BZ 55 and insulin, the stainability as well as the granulation of the alpha cells seemed to decrease in proportion to the duration and degree of the hypoglycemia. The granulation of the beta cells was not appreciably changed in any case.

After a long period of insulin treatment the variability in the stainability of the individual cells in the islets was more noticeable than after a short period of treatment with insulin or BZ preparations. In many instances, two types of alpha cells were noted, on the one hand a type having a small cell-body with intense stainabili-

Fig. 1[1]. Pancreas islet 24 hours after administration of BZ 55 demonstrating the variation in granulation and nuclear size of the alpha cells, and a dilated capillary.
x = scantily granulated alpha cells with large nuclei
o = closely granulated alpha cell with a small nucleus
□ = non-granulated cell with a large nucleus

[1] Stained with chromhematoxylin-Ponceaufuchsin according to Gomori.

ty and such tightly packed granules that the cytoplasm appeared to be homogeneously stained, on the other hand a type with widely spaced granules displaying weaker stainability (Fig. 2), and between these extremes there were transitional stages. In addition to this, there were cells resembling alpha cells as to both localization

Table II.

Nuclear size in μ^2 of pancreatic alpha cells with different degrees of granulation following treatment with BZ 55 and insulin for different lengths of time.

	Close granulation	Scanty granulation	No granulation
BZ 55, 24 hrs	22.2	26.2	26.7
Insulin, 24 hrs	26.7	34.2	30.2
Insulin, 15 days	22.2	28.5	32.9

Fig. 2 a.

Fig. 2[1]. Two pancreas islets (a and b) showing variable granulation and nuclear size in the alpha cells following administration of insulin for 15 days. Note the dilated capillary in section b.
x = scantily granulated alpha cells with large nuclei
o = closely granulated alpha cell with a small nucleus

[1] Stained with chromhematoxylin-Ponceaufuchsin according to Gomori.

and other characteristics but showing no signs of granulation (See top of Fig. 1). Glycogen was not observed in the alpha cells.

The size of the nuclei in the alpha cells has been recorded in Table II and is also illustrated in Figs. 1—2. In general, a smaller nuclear surface was correlated with strong stainability, and the nuclear size increased as the granulation and stainability decreased.

Discussion.

The stainability of the alpha cells in the pancreas after acute insulin shock has previously been studied by several research workers. FERNER (1942), for instance, studied guinea-pigs and found no changes, while HINTEREGGER (1931) and MOHNIKE and HAGEMANN (1951) recorded an increase in the number of alpha cells. After repeated injections of insulin varying results have also been

Fig. 2 b.

reported by FERNER (1942), MILLER (1942), HAMPERL (1951) and HAGEMANN (1952). MILLER, MALCOLM and WURSTER (1956), in studies on lizards, noted increased alpha cell granulation following repeated insulin shocks, but it should be pointed out that lizards develop protracted hyperglycemia following a short period of hypoglycemia. The probable explanation of these contradictory results is, in the first place, that the degree and duration of the hypoglycemia was not checked, and in the second place, that different authors used different staining methods which are difficult to compare, a fact illustrated also in our experiments (Table I).

The changes arising in the alpha cells, especially after treatment with BZ 55, were interpreted by the aforementioned German authors as a sign of inactivity and degeneration. We observed no degenerative changes (pyknosis, etc.) either after BZ or insulin treatment of the duration chosen for our studies. We therefore consider it probable that the decolorization of the alpha cells gradually occurring as the hypoglycemia increases is a reflexion of heightened alpha cell activity. The changes observed in the

nuclear size give added support to this assumption. It cannot be stated with certainty whether this process is equivalent to glucagon secretion or not. There is much to indicate that the alpha cells contain a hyperglycemic principle, and the heightened alpha cell activity during a hypoglycemic state suggests that these cells play a role in the maintenance of a normal blood sugar level.

Summary.

1. After the administration of insulin and of BZ 55 to rabbits identical reduction in the stainability of the alpha cells in the pancreas was obtained; the degree of the changes was closely related to the degree and duration of the hypoglycemia. The nuclear surface of the alpha cells tended to increase as the granulation and stainability decreased, signs probably representing a state of hyperactivity.

2. In our opinion, it is conceivable that the alpha cells play a part in counteracting hypoglycemia and thus are of significance for the blood sugar homeostasis.

Supported by grants from the research fund of the Association of Swedish Life Insurance Companies.

References.

ACHELIS, J. D. and K. HARDEBECK, Dtsch. med. Wschr. 1955. *80*. 1452.
BOVET, D. and P. DUBOST, C. R. Soc. Biol. (Paris) 1944. *138*. 764.
CREUTZFELDT, W. and E. TECKLENBORG, Arch. exp. Path. Pharmak. 1955. *227*. 23.
FERNER, H., Z. mikr.-anat. Forsch. 1938. *44*. 451.
FERNER, H., Virchows Arch. path. Anat. 1942. *309*. 87.
FERNER, H., and W. RUNGE, a) Dtsch. med. Wschr. 1956. *81*. 331. b) Arzneimittelforsch. 1956. *6*. 256.
HAGEMANN, U., Beitr. path. Anat. 1952. *112*. 243.
HAMPERL, H., Virchows Arch. path. Anat. 1952. *321*. 482.
HINTEREGGER, F., Beitr. path. Anat. 1931. *87*. 555.
JANBON, M., P. LAZERGES and J. H. METROPOLITANSKI, Montpellier med. 1942. *21/22*. 489.
KRACHT, J., C. v. HOLT and L. v. HOLT, Endokrinologie 1957. *34*. 129.
LOUBATIÈRES, A., C. R. Soc. Biol. (Paris) 1944. *138*. 766.
LOUBATIÈRES, A., Arch. Int. Physiol. 1946. *14*. 58.
MILLER, R. A. Endocrinology 1942. *31*. 535.
MILLER, R. A., R. MALCOLM and D. H. WURSTER, Endocrinology 1956. *58*. 114.
MOHNIKE, G., and U. HAGEMANN, Klin. Wschr. 1951. *29*. 673.

Acta physiol. scand. 1958. 43. 15—26.

From the Dept. of Pharmacology, University of Lund, Sweden.

The "Desensitizing" Effect of Acetylcholine on the Mammalian Motor End-Plate.

By

J. AXELSSON AND S. THESLEFF.

Received 27 February 1958.

The period of membrane depolarization produced by a persistent concentration of acetylcholine (Ach) in a frog muscle is brief and the motor end-plate region is rapidly and completely repolarized. In the repolarized state the end-plate membrane is refractory *i. e.*, "desensitized" to the neuromuscular transmitter and to applied Ach (THESLEFF 1955).

The kinetics of this desensitization and its recovery processes were recently studied in the frog by ionophoretic microapplication of Ach to the motor end-plate of single muscle fibres (KATZ and THESLEFF 1957). It was shown that an appreciable desensitization of the end-plate membrane occurs (sometimes exceeding 50 per cent) when a relatively small dose of Ach, producing an initial depolarization of 0.5—1 mV, is maintained for 10—20 sec. With higher concentrations of Ach a complete desensitization of the membrane is produced within a few seconds. After withdrawal of Ach the sensitivity of the membrane recovers at a rate which is independent of the duration or degree of desensitization. The hypothesis was put forward that desensitization is due to a reversible change of the receptor molecules from an "effective" to a "refractory" state.

In the present investigation it will be shown that Ach has a marked desensitizing effect also on the mammalian motor end-plate and that considerable species variations in the time course of this process may occur.

Methods.

The ionophoretic micro-application technique has been fully described by DEL CASTILLO and KATZ (1957). A detailed account of the experimental procedure and of the limitations and advantages of this method has been given by KATZ and THESLEFF (1957).

The following muscles were used in the present study: guinea-pig, m. serratus ant.; rabbit, m. serratus post. and the right hemi-diaphragm; rat, the right hemi-diaphragm; cat, m. tenuissimus and m. sternohyoideus. These muscles are thin, they are covered by a comparatively scarce layer of connective tissue and generally have a few muscle fibres with superficial motor end-plates. All muscles were removed from the anaesthetized animal and mounted in a constant temperature bath.

The chamber for the muscle and bathing fluid was a modification of that described by BOYD and MARTIN (1956). The temperature of the bath was normally kept at 34—38° C. The bathing fluid had the composition used by LILEY (1956). Oxygenation was affected by bubbling a 95 % O_2—5 % CO_2 mixture through the fluid immediately prior to its introduction into the muscle bath. The bath held about 30 ml solution which was changed continuously at a rate of about 250 ml/hr.

Single or twin micro-pipettes were used containing a concentrated solution of acetylcholine chloride (Hoffmann—La Roche) or in a few experiments carbachol (Burroughs Wellcome). Individual drug pipettes had tip diameters of less than 1 μ. The discharge of the drug was regulated by "braking" or "releasing" voltages (making the interior of the pipette more negative, or positive, respectively).

The sensitivity of the end-plate membrane was tested by applying series of positive voltage pulses of constant intensity and 10 msec duration to one barrel of the twin pipette. The drug released by these pulses produced in the end-plate membrane transient depolarizations of a few millivolts amplitude and of a rapid time course. These potential changes were recorded with a micro-electrode inserted into the muscle fibre at about 100 microns distance from the point of drug application. The other barrel of the pipette was used to apply a "conditioning" dose *i. e.*, generally a prolonged and steady release of Ach.

The current pulses passing through the drug pipettes were recorded by an a. c. pre-amplifier (frequency response 0.3—10,000 c/sec, decay time constant 0.9 sec at 0.37 maximum height) on the second beam of the oscilloscope. The membrane potential of the muscle fibres was recorded with conventional capillary micro-electrodes as described by FATT and KATZ (1951). In most experiments the resting potential of the muscle fibres was within the range of 70—80 mV.

Results.

In all the various muscles used in this study Ach produced desensitization of the motor end-plate membrane. This was evidenced by the observations that during steady release of Ach the effect of the test pulses successively diminished and that immediately after withdrawal of the conditioning dose a gradual recovery of the response to the test pulses occurred (Fig. 1). Series of records similar to those in Fig. 1 were obtained in about ten experiments with each kind of muscle.

Table I.

Examples of species differences in the effect of varying conditioning doses.

Initial depolarization produced by conditioning dose (mV)	Reduced amplitude of test response (per cent of "unconditioned" response)	Half-time of onset of desensitization (sec)	Half-time of recovery (sec)
Guinea-pig: m. serratus ant.			
1.3	39	10.0	8.0
2.0	33	8.0	11.2
4.5	19	3.8	7.3
5.8	15	2.8	6.3
Rabbit: m. serratus post.			
1.1	55	10.0	7.0
3.2	40	9.0	6.0
3.5	33	5.5	5.0
3.9	22	4.0	5.0
Rabbit: diaphragm			
1.0	39	9.4	5.8
2.0	29	5.1	6.3
4.3	27	3.7	4.7
5.1	19	2.5	5.2
10.0	14	1.4	5.3
Rat: diaphragm			
ca 0.2	40	3.3	3.6
0.4	43	2.5	4.0
0.5	24	1.9	2.8
1.4	21	1.8	3.5
1.7	20	1.0	3.5
Cat: m. tenuissimus			
ca 0.2	33	1.4	2.4
0.3	16	ca 0.8	1.6
0.4	12	ca 0.8	1.9
0.6	6	ca 0.6	2.4
1.0	0	ca 0.5	2.4

Table II.

Mean half-times of recovery from desensitization.

Species and muscle	Number of determinations	Mean half-time of recovery ± S. E. of mean (sec)
Guinea-pig m. serratus ant.	33 (3) [1]	7.2±0.50
Rabbit m. serratus post.	33 (4)	5.0±0.28
diaphragm	29 (3)	4.9±0.14
Rat diaphragm	57 (4)	3.4±0.16
Cat m. tenuissimus	50 (6)	2.8±0.17

[1] Number of muscles

The onset and the recovery of the desensitization process, as revealed by the decreasing amplitude of the test response, followed an approximately exponential curve. From the steepest parts of these curves the half-time of onset and recovery could be determined and it was thereby possible to obtain an estimate of the time course of the desensitization produced by varying conditioning doses (Table I).

As previously demonstrated in the frog (KATZ and THESLEFF 1957) it was also in the present investigation observed that the degree and speed of desensitization was graded and increased with the conditioning dose while the rate of recovery was not greatly altered (Table I). Fig. 1 and Table I also show that marked species differences exist in the relations between conditioning dose, final intensity of desensitization and time course of onset and recovery. In the guinea-pig a conditioning dose producing an initial membrane depolarization of 1—2 mV reduced the test response by about 50 per cent while in the cat the same degree of desensitization was generally caused by a conditioning dose producing only one tenth as much depolarization. The rate of onset of desensitization showed similar variations, in the guinea-pig and the rabbit the half-time of onset of desensitization generally exceeded 1—2 sec while half-times of less than one second were commonly observed in the rat diaphragm and particularly in the tenuissimus and sternohyoideus muscle of the cat.

The recovery rate was independent of the conditioning dose and the degree of desensitization. However, marked species differences occurred, see Table II. It is of interest that the half-

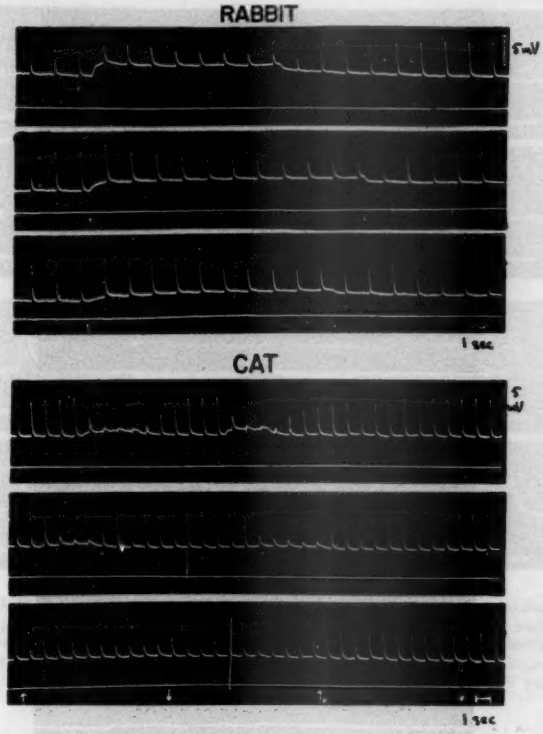


Fig. 1. Desensitization produced by different conditioning doses of Ach at a single end-plate spot. Upper part: rabbit; m. serratus post. Lower part: cat; m. tenuissimus. The onset and with-drawal of the conditioning dose is marked with arrows and its strength increases successively from down upwards. Current pulses through pipette are registered in lower trace of each recording. Monitor calibration (5 mV scale) upper part $= 9.1 \times 10^{-7}$ A. Lower part $= 2.1 \times 10^{-7}$ A.

time of recovery appeared to be related to the susceptibility of the muscle to the desensitizing effect of Ach, *i. e.* that muscles in which desensitization was most readily produced also had the fastest rate of recovery. After prolonged and large conditioning doses recovery occasionally was slow and/or incomplete. Whether this was due to a second component in the recovery process or to an artifact could not be decided.

In the rat diaphragm and in m. tenuissimus of the cat desensiti-

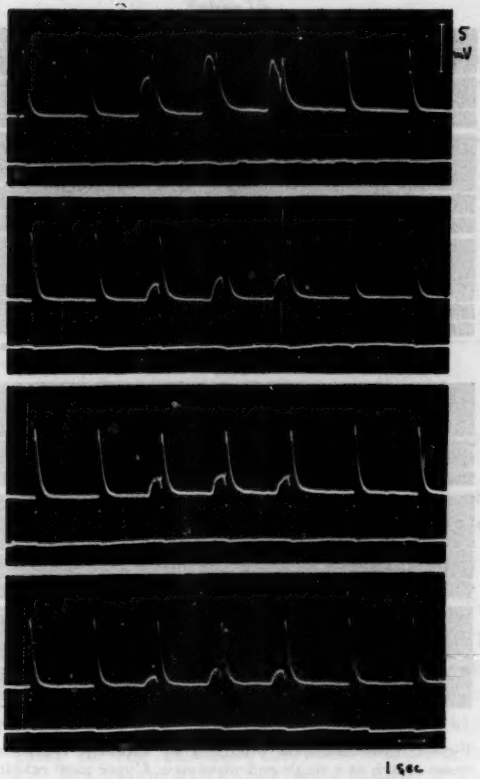


Fig. 2. Rat diaphragm. The effect of a conditioning pulse of 0.5 sec duration and varying intensity on a test pulse (10 msec duration) succeeding it with a delay of 50 msec. Monitor calibration (voltage scale) = 7×10^{-7} A.

zation was frequently produced so rapidly and with such small conditioning doses that with the aforementioned procedure it was impossible to get a proper estimate of dose-response relations. The method adopted in a number of experiments was therefore to use a conditioning pulse of short duration and variable intensity and to allow the test pulse to succeed it with a known delay. Results obtained in the rat diaphragm muscle with this procedure are shown in Fig. 2 which demonstrates that a conditioning pulse of

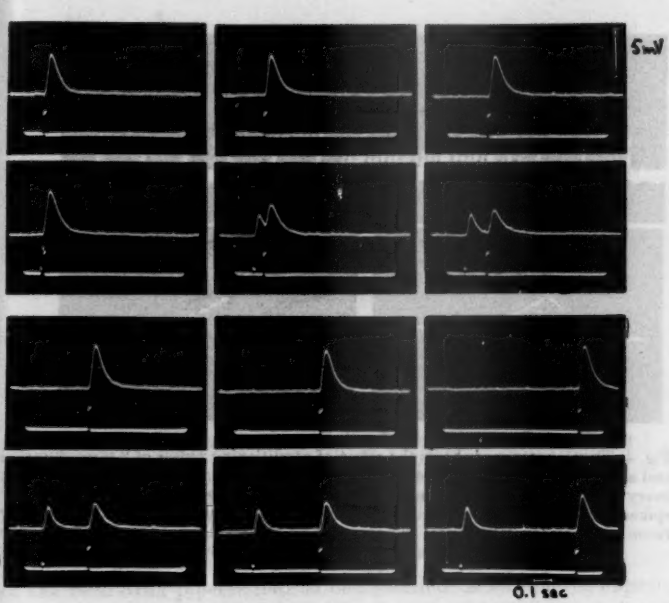


Fig. 3. Cat: m. tenuissimus. The effect of a conditioning pulse of 20 msec duration on a test pulse (10 msec duration) following it at varying delay. Upper and third row: the test pulse alone. Second and fourth row: the test pulse preceded by the conditioning pulse. Note that the conditioning pulse produces an almost immediate reduction in the amplitude of the test response. Monitor calibration (5 mV scale) = 1.1×10^{-7} A.

0.5 sec duration produces a significant reduction in the subsequent test response. When similar experiments were made in the tenuissimus muscle of the cat the astonishing observation was made that a conditioning pulse having a duration of 20 msec and causing only a few millivolts depolarization produced a marked desensitization of the end-plate membrane. This is shown in Fig. 3 in which such a short conditioning pulse produces an almost immediate and marked desensitization that lasts for more than one second.

Experiments were also made in the tenuissimus muscle of the cat with conditioning pulses of 1—2 msec duration. Such brief pulses produce in the end-plate membrane depolarizations with a rapid time course (total duration 20—30 msec) and about one millivolt amplitude. As judged from the amplitude of a subsequent test response these conditioning pulses do not produce desensiti-

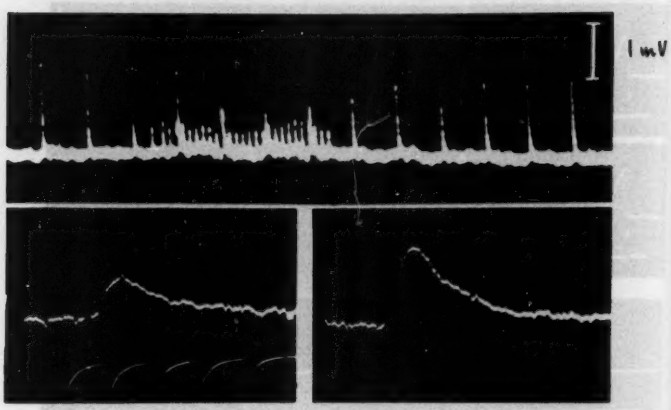


Fig. 4. The upper record shows the effect of conditioning pulses of 2 msec duration and a frequency of 6/sec on a test response produced by a pulse of 1.5 msec duration every second. Note spontaneous miniature e. p. p.'s shown as faint and irregular spikes with an amplitude similar to the conditioning pulse response. In the lower records are to the left shown the response to the conditioning pulse and to the right the response to the test pulse. Time 10 msec.

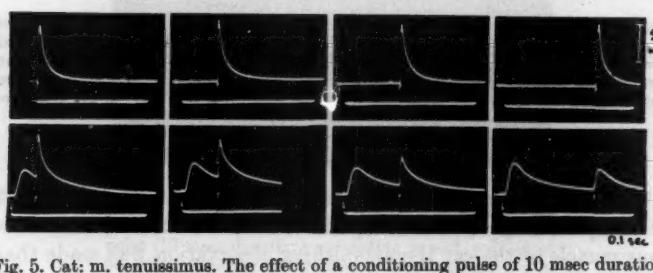


Fig. 5. Cat: m. tenuissimus. The effect of a conditioning pulse of 10 msec duration on a test pulse (10 msec duration) following it at varying delay. Upper row: the test pulse alone. Lower row: the test pulse preceded by the conditioning pulse. Note that the conditioning pulse besides reducing the amplitude of the test response also alters its time course. The conditioning and test doses are applied by separate single barrel pipettes. Monitor calibration (5 mV scale) = 5.9×10^{-7} A.

zation of the membrane. When, however, several of them are applied, at a frequency of 3—6/sec a considerable desensitization develops (Fig. 4).

The rapidity with which desensitization developed in the rat diaphragm and particularly in the tenuissimus muscle of the cat was surprising and before accepting such a finding any peculiarity

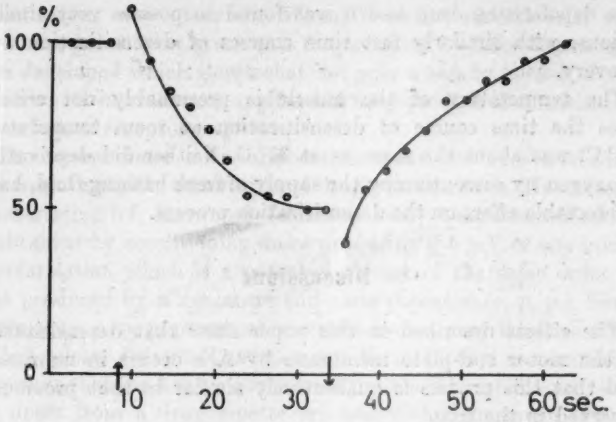


Fig. 6. Guinea-pig: m. serratus ant. Amplitude of test responses during and after application of a conditioning dose producing an initial depolarization of 1.3 mV. The onset and withdrawal of the conditioning dose are marked by arrows. For explanation see text.

of the twin drug pipette had to be excluded. A number of experiments were therefore made with two separately manipulated single-barrel pipettes. When the tips of the two drug pipettes were brought close to the same sensitive spot drug interaction occurred and records such as those shown in Fig. 5 were obtained. From these records it is evident that the time course of desensitization produced by two separate drug pipettes is rather similar to that found when a twin pipette was used and that consequently the suggestion of an artifact due to barrel interaction can be dismissed.

In the guinea-pig, the rabbit, occasionally in the rat but never in the cat it was observed that a test pulse superimposed at the beginning of a small conditioning depolarization produced more than a simple additive effect. In those instances a withdrawal of the conditioning dose produced an immediate and sharp reduction in the height of the test response, see Fig. 6. These effects were similar to those previously described in the frog (KATZ and THESLEFF 1957) and are presumably due to an "S-shaped" dose-effect relation. As in the frog, the conditioning dose, besides altering the amplitude of the test response, also generally produced a slowing of its time course (Fig. 5).

In addition to Ach, carbachol was in a few experiments used

as a depolarizing drug and it was found to possess very similar actions, with similarly fast time courses of desensitization and recovery.

The temperature of the muscle is presumably not critical since the time course of desensitization at room temperature (22° C) was about the same as at 37° C. Neither did deprivation of oxygen by discontinuing the supply of fresh bathing fluid, have a detectable effect on the desensitization process.

Discussion.

The effects described in this paper show that desensitization of the motor end-plate membrane by Ach occurs in mammals, and that this process is qualitatively similar to that previously observed in the frog.

It is noteworthy that species variations exist in the time course of the desensitization process. One explanation for such differences would be variations in the diffusion time of the drug caused by connective tissue and/or the spatial distribution of Ach receptors and cholinesterase. However, since large variations occurred in the initial depolarization produced by equipotent desensitizing doses it is more likely that it is the rate of the onset and recovery processes in desensitization that varies between species.

The possibility that the time course and degree of desensitization even within one species differs from one muscle to another has not been systematically investigated. The observation that the desensitization processes in the diaphragm muscle and in m. serratus post. of the rabbit and in m. tenuissimus and sternohyoideus of the cat were quite similar may possibly be an indication that such variations are small.

The most surprising feature in this investigation was the rapidity with which desensitization often developed in the rat diaphragm muscle and particularly in m. tenuissimus of the cat. Whereas in the guinea-pig and rabbit a steady efflux of Ach was needed during several seconds to produce desensitization, the same effect was obtained in the rat and in the cat by smaller conditioning doses and within a few tenths of a second.

In the tenuissimus muscle of the cat conditioning pulses of 10—20 msec duration produced considerable desensitization of the end-plate. With conditioning pulses of 1—2 msec duration no de-

sensitization was observed. When, however, several brief conditioning pulses were given at a frequency of 3—6/sec desensitization developed which shows that not only a steady release of Ach but also its intermittent application may produce desensitization of end-plate receptors.

The ionophoretic micro-application technique does not allow one to estimate the attained local drug concentration. However, it is interesting (cf. KATZ and THESLEFF 1957), that desensitization is obtained by conditioning doses producing 0.5 mV or less initial depolarization which is a potential change of the same order as that produced by a miniature end-plate potential (e. p. p.). Since a miniature e. p. p. presumably results from a very localized form of Ach release (cf. DEL CASTILLO and KATZ 1956) it is reasonable to assume that the Ach concentrations produced by small conditioning doses from a drug pipette are well within the range of concentrations of Ach that occur during an ordinary e. p. p. Consequently, one could expect that in the cat and probably also in the rat the concentration of Ach released by one or a few successive motor nerve impulses would be sufficient to produce desensitization of the post-junctional end-plate membrane. The possibility therefore exists that the desensitization process could, at least in part, be responsible for the well known decline in amplitude of successive e. p. p.'s observed in muscles from these two species (ECCLES, KATZ and KUFFLER 1941, LILEY and NORTH 1953, LUNDBERG and QUILISCH 1953).

Summary.

1. A micro-application technique has been used to study the desensitization which acetylcholine produces at the mammalian motor end-plate. Acetylcholine was released by ionophoresis from a twin pipette near sensitive spots in the end-plate region of a single muscle fibre, and the resulting potential changes were recorded by an intracellular electrode. Isolated muscles mounted in a constant temperature bath from the following mammalian species were used: guinea-pig, rat, rabbit and cat.

2. In all the investigated muscles a steady release of acetylcholine produced a loss of sensitivity in the end-plate membrane. The degree and the speed of onset of desensitization was graded and increased with the applied concentration of acetylcholine while the rate of recovery was relatively constant.

3. Marked species variations were observed in the intensity and time course of desensitization. In the guinea-pig and the rabbit it was necessary to have a steady efflux of acetylcholine producing an initial depolarization of a few millivolts during several seconds to obtain desensitization. In the rat and the cat the same effect was generally achieved within a few tenths of a second by concentrations of acetylcholine producing less depolarization. The half-times of recovery varied in a similar way.

4. In the tenuissimus muscle of the cat a conditioning pulse of 10—20 msec duration causing only a few millivolts depolarization frequently produced an almost immediate desensitization lasting for more than one second. No desensitization was observed after single conditioning pulses of 1—2 msec duration but when several such pulses were given at a frequency of 3—6/sec considerable desensitization developed.

5. It is suggested that the concentrations of acetylcholine which produce desensitization are, at least in certain mammalian muscles, within the range of concentrations normally produced at the end-plate by a motor nerve impulse, and that consequently desensitization may develop during repetitive motor nerve stimulation.

The expenses of this investigation were aided by grants from the Swedish Medical Research Council, The Muscular Dystrophy Association of America, Inc. and the Air Research and Development Command, United State Air Force, through its European Office. We are indebted to Miss E. Adler for valuable technical assistance.

References.

Boyd, I. A. and A. R. Martin, J. Physiol. 1956. *132*. 61.
del Castillo, J. and B. Katz, Progr. Biophys. and Biophys. Chem. 1956. *6*. 121.
— — Proc. roy. Soc. B. 1957. *146*. 339.
Eccles, J. C., B. Katz and S. W. Kuffler, J. Neurophysiol. 1941. *4*. 362.
Fatt, P. and B. Katz, J. Physiol. 1951. *115*. 320.
Katz, B. and S. Thesleff, J. Physiol. 1957. *138*. 63.
Liley, A. W., J. Physiol. 1956. *132*. 650.
— and K. A. K. North, J. Neurophysiol. 1953. *16*. 509.
Lundberg, A. and H. Quilisch, Acta physiol. scand. 1953. *30*. 111.
Thesleff, S., Acta physiol. scand. 1955. *34*. 218.

Acta physiol. scand. 1958. 43. 27—50.

From the Department of Pharmacology, University of Gothenburg, Gothenburg, Sweden.

The Mechanism of the Vasodilator Effect of Adrenaline.

III. Influence of Adrenaline, Noradrenaline, Lactic Acid and Sodium Lactate on the Blood Pressure and Cardiac Output in Unanesthetized Rabbits.

By

LENNART LUNDHOLM.

Received 27 February 1958.

In the two earlier papers in this series (LUNDHOLM 1957 a, b), experimental results were presented which suggested that the vasodilator effect of adrenaline in skeletal muscle and the fall of blood pressure that, particularly in anesthetized animals, accompanied it were due to its stimulatory action on the lactic acid production in skeletal muscle. However, several investigators have also observed a nervous reflex vasodilatation after administration of adrenaline. Since, on intra-arterial infusion of that substance, the vasodilator effect occurs in both denervated and totally isolated extremities (DALE and RICHARDS 1927, WHELAN 1952), it cannot be due solely to a nervous mechanism. Yet it has been demonstrated conclusively that adrenaline, on intravenous infusion, may inhibit sympathetic tone via different mechanisms. HEYMANS, BOUCKAERT and WIERZUCHOWSKI (1937), in cross-circulation experiments, thus showed that it could pro-

duce vasodilatation in isolated perfused extremities by sympathetic inhibition, not only via the baroceptors in the carotid and aortic bodies but also via receptors in more distal segments of the aorta. Even local application of adrenaline in the region of the carotid body in a concentration that contracted the vessel, induced vasodilatation (HEYMANS and VAN DEN HEUVEL-HEYMANS 1950). GRUHZIT, FREYBURGER and MOE (1954) and DÖRNER (1953, 1956) found that in the region of distribution of the thoracic aorta, too, there existed receptors the stimulation of which with adrenaline or noradrenaline led to vasodilatation in cross-perfused extremities. MARRAZZI (1939) and LUNDBERG (1952) reported that adrenaline inhibited the transmission in sympathetic ganglia; but according to BURN and BÜLBRING (1942) and MALMÉJAC (1955) the synaptic transmission was inhibited in higher concentrations but was facilitated in lower concentrations.

In experiments conducted by DALE and RICHARDS (1927), EULER (1938) and TAYLOR and PAGE (1951) adrenaline, when injected into the cerebral vessels, elicited a fall of blood pressure and vasodilatation. EULER attributed this to cerebral ischemia caused by adrenaline's constrictor effect on the cerebral vessels, but TAYLOR and PAGE postulated receptors in the brain that were specially susceptible to adrenaline and noradrenaline. There is no reason to doubt that under certain experimental conditions these different mechanisms may lead to reflex vasodilatation; but the question is to what degree this reflex dilatation has a bearing on the vasodilator effect of adrenaline in the intact, unanesthetized organism. In the experiments that have demonstrated reflex vasodilatation, comparatively large doses of adrenaline have been administered and a cross-circulation technique has been used. The question arises whether the tendency to vasodilatation that results from reduced sympathetic tone is not counteracted by the direct stimulatory effect of adrenaline on the vascular muscle layer in the intact organism. If the reflex vasodilatation were of major significance in the vasodilator and depressor effect of adrenaline, then vasodilatation might be expected to arise also in other vascular areas than the skeletal muscle, the liver and the heart. ENGELHORN (1956), on recording the action potentials from the sympathetic nerves to the kidneys, thus found that adrenaline in depressor doses increased the activity in those fibers, whereas pressor doses reduced the activity, the latter in agreement with the findings reported by GERNANDT, LILJESTRAND and ZOTTERMAN (1946).

Since, however, only a vasoconstrictor effect of adrenaline was found on the renal vessels the implication is that, in that vascular area at least, the reflex vasodilatation is completely overshadowed by the direct stimulatory action on the vascular smooth muscle. The results communicated by SWAN (1951) point to such a competition between the reflex vasodilator and the direct vasoconstrictor effect. SWAN found in human subjects that an adrenaline infusion which constricted the cutaneous vessels was followed by a flush, probably of nervous origin since it failed to appear after sympathectomy.

Lastly, most workers who have studied the reflex vasodilator effect of adrenaline have conducted their experiments under chloralose or barbiturate anesthesia, which more or less strongly inhibit the lactic acid producing action of adrenaline (LUNDHOLM 1957 b) and hence tend to weaken the direct dilator effect in relation to the reflex one.

In earlier experiments the depressor effect was found to be slight in unanesthetized animals, but peripheral vasodilatation was demonstrable since even at an unchanged or slightly elevated mean blood pressure the cardiac output rose considerably, indicating a fall in the total peripheral resistance. This was taken to imply that the blood pressure regulation was better in unanesthetized than in anesthetized animals (LUNDHOLM 1957 b). It could therefore be assumed that the elevated cardiac output, after at least small doses of adrenaline, was of reflex origin and secondary to the vasodilatation induced via stimulation of the lactic acid production in skeletal muscle. This assumption was further supported by ENGELHORN's report (1956) that the impulse activity rose in the sympathetic nerves after adrenaline in doses having a depressor effect.

If the direct stimulatory action of adrenaline on the heart were accompanied by reflex dilatation in different vascular areas, then it should be manifested chiefly after adrenaline in doses tending to elevate the mean blood pressure. In order, therefore, to clarify as far as possible the effect produced on the cardiac output by adrenaline's stimulation of the lactic acid production, the smallest practicable dose should be administered. — While stimulation of the lactic acid production in cats increased almost linearly with the adrenaline dose (LUNDHOLM 1957 b), even 0.2 μg adrenaline/kg/min in rabbits had a maximal stimulatory effect (LUNDHOLM 1949). CORI, CORI and BUCHWALD (1930) found that the threshold

dose for the pressor effect of adrenaline amounted to 0.8 μg/kg/min in unanesthetized rabbits. In rabbits under chloralose anesthesia the corresponding value was 1.1 μg/kg/min (LUNDHOLM 1949). In rabbits the pressor threshold dose accordingly seemed to be quite different from that having a maximal stimulatory action on the lactic acid production — which was not the case in cats.

It was also worth while investigating any effect of adrenaline on the rabbit's blood circulation, since its depressor effect was demonstrable in that animal only in some experiments (HARVEY and NICKERSON 1953). Inasmuch as the stimulation of the lactic acid production was at least as great in rabbits as in cats, adrenaline might be expected regularly to have a vasodilator effect in rabbits too.

Methods.

Due to the profound influence of various anesthetics upon the lactic acid producing and the circulatory effects of adrenaline (LUNDHOLM 1957 b), it was found inadvisable to perform the experiments on anesthetized animals. Incidentally, the stimulatory action of adrenaline, at least in small doses, on the cardiac output has been demonstrated only in unanesthetized human subjects. The experiments were therefore conducted on unanesthetized rabbits weighing between 2.5 and 5 kg. In small rabbits preparation was technically difficult and the animals did not tolerate the blood losses so well when samples were taken. After 15 hours without food, a small cannula was introduced into one marginal vein of the ear and the rabbits were anesthetized with a 2 per cent solution of evipan sodium. Following local anesthesia with 2 per cent lidocain the jugular vein was dissected free on the right side of the neck. Into one of its branches was inserted a fine polyethylene catheter (outer diameter 1.3 mm; thickness of wall 0.4 mm). A steel wire guide 0.3 mm in caliber stiffened the catheter sufficiently for introduction into the right ventricle. This guide was slightly curved at the tip, enabling the catheter to be directed as required by appropriate rotation. When the heart beats were perceptible in the catheter, the position in the right ventricle was checked fluoroscopically, the guide then being withdrawn and the catheter filled with 1 per cent heparin solution. Through another branch of the jugular vein a similar catheter was advanced down to the

junction of the subclavian vein. The intravenous infusions were given via this latter catheter.

In the first few experiments a plastic catheter was inserted into the right common carotid for recording of the blood pressure. In subsequent experiments it was introduced instead into the right external carotid and advanced to a point about 1 cm above the origin of the internal carotid. The purpose of this modification was as far as possible to avoid affecting the baroceptors in the carotid body. However, there was no demonstrable difference between the results with the two methods. The plastic catheter had an outer diameter of 2.4 mm and an inner one of 1.0 mm. Its terminal part, 2 cm in length, was heated and fashioned to a suitable size for the various arteries.

To the arterial catheter was connected, about 4 cm from its tip, a Y-shaped cannula, one branch of which was joined to a somewhat larger catheter communicating with a mercury manometer for blood pressure recording, and the other to a smaller catheter for blood sampling. The experimental animal then received 1 ml of 5 per cent heparin solution intravenously.

After meticulous hemostasis the neck wound was closed and the catheters sutured in the skin, after which they were attached with adhesive tape to the right ear, passing in a gentle curve from the neck to the ear so that the animal could move its head freely without dislodging them. A dressing was applied to the neck wound and attached with adhesive tape, though the latter had to be somewhat loose, for otherwise inspiratory stridor developed.

In some experiments the thorax was depilated, after which two electrodes were attached in precordial positions by a rubber band round the thorax. By this means the heart rate was recorded directly on an electrocardiograph. The respiration rate was recorded with a stethograph round the thorax and abdomen, and a tambour.

When the general anesthesia commenced to wear off, the rabbit was placed in the respiration chamber of a metabolic apparatus that has previously been described in detail (LUNDHOLM 1949). The different catheters were then led out of the chamber via an airtight device, and connected to the various recording instruments.

When the animal had recovered from the anesthesia, 60—90 minutes after preparation, and its oxygen consumption had been constant for at least 30 minutes, the experiment began.

The different catheters for blood sampling were filled with 1

per cent heparin solution. In withdrawing blood samples this solution was first aspirated together with a further 1 ml blood, after which 1.5 ml of arterial blood was taken for lactic acid assay and 1.5 ml of arterial and venous blood (from the right ventricle) for gas analysis.

The lactic acid content was determinated *ad modum* FRIEDEMAN and GRAESER (1933); the CO_2 and O_2 contents of the blood by van Slyke's manometric method on 0.5 ml blood (PETERS and VAN SLYKE 1932).

The experimental procedure was as follows. When the O_2 consumption had become constant, the basal oxygen consumption was recorded in 5-minute periods for 30 minutes. During the last one or two of these periods blood samples were taken over the course of 2 minutes from the right ventricle and from the maxillary artery. At the outset of the experimental series blood samples were withdrawn midway through the last 5-minute period, and the oxygen consumption over that period was taken as a basis for calculation of the cardiac output during it. However, the variation in oxygen consumption between different 5-minute periods proved to be so great that it was better to withdraw the samples midway between two periods and to take the mean value for the 10-minute period when calculating the cardiac output. The apparent increase in oxygen consumption that arose from the withdrawal of blood — with the rabbit in a closed system the volume changes of which were recorded — was corrected in calculating the O_2 consumption for the relevant 10-minute period. The blood for gas analysis was taken in a paraffinized glass syringe containing a small drop of mercury. Before analysis of the blood for CO_2 and O_2 the plasma and the corpuscular fraction were mixed, with the help of the drop of mercury.

After blood sampling for determination of the basal cardiac output, the appropriately diluted solutions of the various substances were infused intravenously at a constant rate of 0.3 ml/min with the aid of an infusion apparatus. Generally the infusion time amounted to 60 minutes, but in a few cases 35 minutes. The solvent consisted of sterile pyrogen-free salt solution. In the experiments with adrenaline and noradrenaline this solution was saturated with N_2 to prevent oxidation of the drugs. In the lactic acid experiments dl-lactic acid was used, and in the experiments with sodium lactate the lactic acid was neutralized with NaOH to pH 7.3.

Table I.

The effect of 0.3 μg/kg/min of l-adrenaline on the cardiac output and the lactic acid contents of the blood.

Weight kg	Basal period				Infusion 25—35 min		Infusion 50—60 min	
	O_2-consumpt. ml/min	arterio-venous O_2-diff. vol. %	cardiac output ml/min	lactic acid mg %	cardiac output per cent	lactic acid mg %	cardiac output per cent	lactic acid mg %
3.6	34.6	4.6	753	28.0	—	—	137	62.5
3.8	34.6	4.6	752	6.5	113	32.3	—	—
3.2	34.4	4.6	748	17.4	219	44.3	105	51.8
3.6	30.6	5.7	537	5.8	101	24.5	103	20.4
4.0	29.8	3.6	828	12.2	231	27.7	170	36.7
4.6	31.8	5.5	578	9.5	115	15.3	119	20.1
3.45	38.6	4.9	788	14.5	101	22.6	129	23.3
3.1	25.6	4.2	610	7.2	114	13.4	84	12.6
2.8	23.3	6.7	348	17.7	140	43.9	122	50.0
3.4	31.3	8.0	391	14.6	210	44.3	267	50.3
3.2	30.7	5.1	602	15.3	160	22.1	134	21.7
3.2	31.0	5.4	574	15.3	148	33.3	157	22.2

Blood samples were taken for determination of the cardiac output and for lactic acid assay 25—35 minutes and 50—60 minutes after the start of infusion.

Results.

The results of the different experimental series are reported in Tables I—III and Figs. 1—5. In the figures are recorded the means from the different experiments. The O_2 consumption and cardiac output during the infusion are given in per cent of the basal value. The blood pressure is indicated in mm of mercury; the lactic acid content of the blood, as the increase in mg per cent over the basal value. Changes in the heart and respiration rates are indicated in the text.

Control experiments. In four experiments saline alone was infused. As will be evident from Fig. 1, no appreciable changes arose in blood pressure, cardiac output, oxygen consumption, or lactic acid content of the blood. Whether the fall in cardiac output that occurred towards the end of the infusion period indicated the onset of more basal conditions, or was due to random factors, could not

Table II.

The effect of 5—10 mg/kg/min of dl-lactic acid on the cardiac output and the lactic acid contents of the blood.

Weight kg	Dose mg/kg per min	Basal period				Infusion 25—35 min		Infusion 50—60 min	
		O_2-consumpt. ml/min	arterio-venous O_2-diff. vol. %	cardiac output ml/min	lactic acid mg %	cardiac output per cent	lactic acid mg %	cardiac output per cent	lactic acid mg %
3.6	10	27.7	4.2	660	24.5	119	56.6	—	—
4.6	10	28.1	5.7	493	8.1	150	48.5	—	—
3.5	10	29.8	6.1	489	7.0	220	38.2	104	45.8
3.6	10	27.9	2.7	1,033	11.5	148	59.3	132	41.8
3.5	10	27.7	5.0	554	7.6	125	40.8	—	—
2.3	10	28.7	6.8	422	12.2	92	48.0	98	70.1
2.6	10	22.8	5.6	407	13.8	105	62.3	110	95.5
3.0	5	28.2	3.5	806	11.7	81	32.4	100	34.5
2.6	5	21.3	4.5	473	25.6	141	59.9	102	71.9
2.6	5	24.1	6.3	383	24.7	116	40.1	110	52.6
2.2	5	23.2	3.9	595	16.1	111	36.8	70	45.5
2.3	5	22.1	4.9	451	12.5	190	29.1	117	36.5

Table III.

The effect of 1: control infusion of 0.3 ml 0.9 % NaCl/min, 2: 10 mg/kg/min of dl-lactic acid as sodium salt, 3: 0.5 µg/kg/min of l-noradrenaline on the cardiac output and the lactic acid contents of the blood.

	Weight kg	Basal period				Infusion 25—35 min		Infusion 50—60 min	
		O_2-consumpt. ml/min	arterio-venous O_2-diff. vol. %	cardiac output ml/min	lactic acid mg %	cardiac output per cent	lactic acid mg %	cardiac output per cent	lactic acid mg %
Control infusion	3.4	34.0	8.7	391	9.1	118	10.5	99	7.8
	3.2	22.5	5.8	388	7.5	115	7.6	98	7.4
	4.1	35.7	6.8	525	11.6	85	10.9	83	9.4
	4.0	31.3	6.6	473	23.8	92	27.2	74	27.5
Na-lactate	3.0	23.2	6.8	341	12.3	118	42.7	126	50.4
	3.0	21.5	5.8	371	8.0	105	40.3	90	68.4
	2.1	22.2	5.8	383	5.8	112	42.3	156	45.7
	2.4	28.0	7.0	400	13.0	103	35.4	102	46.3
Nor-adren-aline	2.4	25.9	6.9	375	9.3	104	15.9	124	16.8
	3.0	23.8	5.0	475	7.2	102	8.6	90	8.3
	2.5	24.0	7.6	316	8.3	103	11.3	75	11.1
	5.0	38.5	4.3	857	21.1	112	25.9	111	21.7
	4.9	44.2	5.5	804	14.0	113	19.5	110	23.6
	4.4	33.5	4.1	817	9.5	—	—	118	13.9

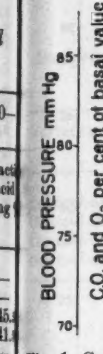


Fig. 1. Co
oxygen co
basal valu

be decid
physiolo
constant
such hac

Adren
ine, Bio
it is evi
cent aft
time bei
output v
the bloo
lactic ac
cent abo
by 6—8
ine expe
the cont

The ba
the first
1—2 bea
rise to 24
Thus it
be discu

The r
minute t
infusion.

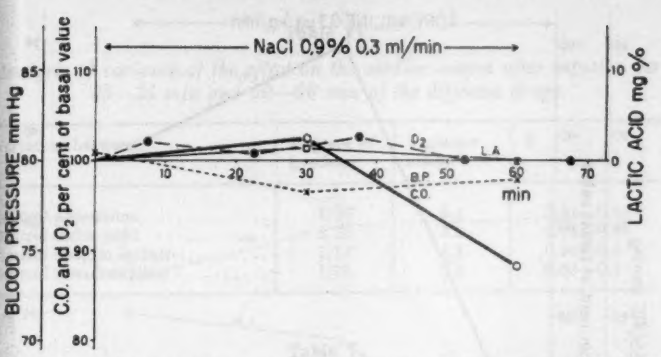


Fig. 1. Control infusion of 0.9 per cent NaCl. B. P.: Blood pressure mm Hg. O_2: oxygen consumption per cent of basal value. C. O.: cardiac output per cent of basal value. L. A.: increase of lactic acid content of the blood above basal value.

be decided. The control experiments showed that the recorded physiological magnitudes could be expected to remain relatively constant during the infusion period, and that the salt solution as such had no effect.

Adrenaline. In 12 experiments, adrenaline (synthetic l-adrenaline, Bios) was infused in a dose of 0.3 μg/kg/min. From Fig. 2 it is evident that the cardiac output increased by about 50 per cent after 30 minutes infusion, the blood pressure at the same time being virtually unchanged. Even after 60 minutes the cardiac output was still about 40 per cent higher than the basal level, and the blood pressure had fallen by an average of 3 mm Hg. The lactic acid content of the blood rose by a maximum of 20 mg per cent above the basal value, and the oxygen consumption increased by 6—8 per cent. The increase of the cardiac output in the adrenaline experiments was statistically probable by comparison with the controls (Table IV).

The basal heart rate averaged 236 beats per minute, and during the first 20 minutes of the infusion the increase amounted only to 1—2 beats. Not until 30 minutes had elapsed did the heart rate rise to 244 per minute, and after 60 minutes it was 247 per minute. Thus it showed no increase early in the infusion, which fact will be discussed later on.

The respiration rate increased from a basal value of 50 per minute to 54 and 55 per minute respectively after 30 and 60 minutes infusion.

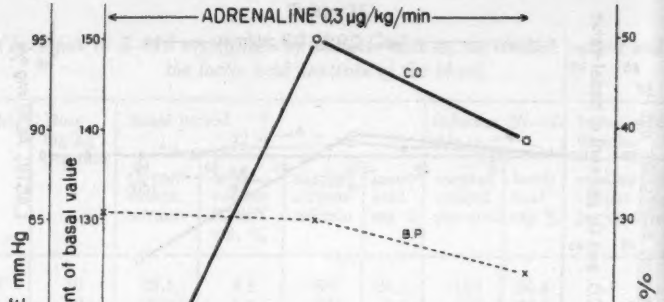


Fig. 2. The effect of adrenaline on: blood pressure (B. P.): oxygen consumption (O_2): cardiac output (C. O.): lactic acid content of the blood (L. A.).

It has been generally assumed that adrenaline is devoid of vasodilator effects in the rabbit. HARVEY and NICKERSON (1953), who discussed this question, pointed out that both in earlier experiments and in their own, adrenaline reversal had been demonstrable after adrenolytics in rabbits; and GIRLING (1951) found that on injection of adrenaline into the femoral artery, low doses produced vasodilatation.

In these experiments on rabbits, in which the cardiac output rose in spite of an unchanged blood pressure, the total peripheral resistance had decreased by 25 and 24 per cent respectively after 30 and 60 minutes infusion.

The circulatory effects of adrenaline in the unanesthetized rabbit were thus in accord with those previously observed in unanesthetized cats (LUNDHOLM 1957 b). A comparison of its effects on the rabbit and the human circulation (Table V) also reveals conspicuous similarities. The unanesthetized individual, as pointed out earlier (LUNDHOLM 1957 b), shows no reaction or only a transient fall of blood pressure after adrenaline in small doses (ALLEN,

Table IV.

Analysis of variance of the effect on the cardiac output after infusion for 25—35 min and 50—60 min of the different drugs.

Variation between:	Degrees of freedom	Variance ratios	P
Control-adrenaline	1/26	6.5	0.01—0.05
Control-lactic acid	1/25	4.5	0.01—0.05
Control-sodium lactate	1/12	4.1	0.05—0.2
Control-noradrenaline	1/15	2.3	0.05—0.2

Table V.

Comparison between the hemodynamic response of the rabbit to adrenaline, noradrenaline and lactic acid and the response of man (values of Goldenberg, Pines, Baldwin, Greene, *and* Roh *(1948)) to adrenaline and noradrenaline. In the rabbit the effect was determined after intravenous infusion for 25—35 min of the different drugs. The response of man was measured after intravenous infusion for 12 min of adrenaline ($n = 6$) and after 15 min of infusion of noradrenaline ($n = 9$). All values are expressed as per cent of the basal values.*

Effect	Rabbit			Man	
	Adrenaline	Noradrenaline	Lactic acid	Adrenaline	Noradrenaline
O_2-consumption	110	103	109	123	112
Arterio-venous O_2-diff.	80	97	85	69	118
Cardiac output	150	107	132	180	97
Blood pressure	101	114	100	106	128
Pulse	101	94	89	118	77
Stroke volume	151	120	175	154	124
Peripheral resistance	75	107	82	60	135

Barcroft and Edholm 1946; Barnett, Blacket, Depoorter, Sanderson and Wilson 1950); instead, the cardiac output rises and offsets the fall in peripheral resistance.

Lactic Acid. For duplicating as far as possible, by lactic acid infusion, the increase in the lactic acid content of the blood after adrenaline, it would have been best to infuse l(+) lactic acid — sarco lactic acid — which is metabolized about four times faster than d(—) lactic acid (Lundholm, to be published). It was found difficult, however, to prepare the free acid from zink-l(+) lactate

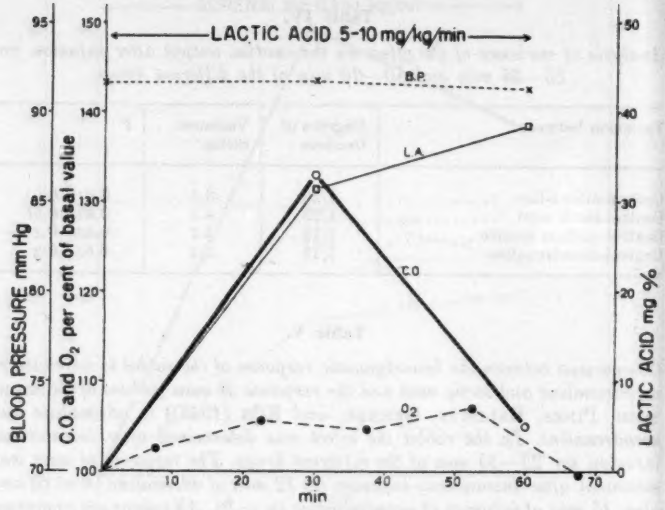


Fig. 3. The effect of lactic acid on: blood pressure (B. P.): oxygen consumption (O_2): cardiac output (C. O.): lactic acid content of the blood (L. A.).

without contamination of zink or without partial racemization of the acid. But since it was chiefly the circulatory effects of lactic acidosis that were to be investigated, the racemic form could be assumed to have the same effect as l(+) lactic acid.

In seven experiments, 10 mg dl-lactic acid/kg/min was infused intravenously, this amount earlier having proved to correspond to the maximal lactic acid producing effect of adrenaline (LUNDHOLM 1949). Since, however, the racemic form was used in these experiments, the lactic acid elevation in the blood was greater than that in the adrenaline experiments. In five experiments, therefore, only 5 mg dl-lactic acid/kg/min was infused, and in these cases the increase of the lactic acid content was more like that after adrenaline. No difference emerged in the circulatory effects of the various amounts of lactic acid, and the experiments have therefore been combined in Fig. 3. From the latter it will be seen that after 30 minutes infusion the cardiac output had risen by an average of 33 per cent, but after 60 minutes by only 5 per cent. The difference between the lactic acid and the control experiments was statistically probable (Table IV). The blood

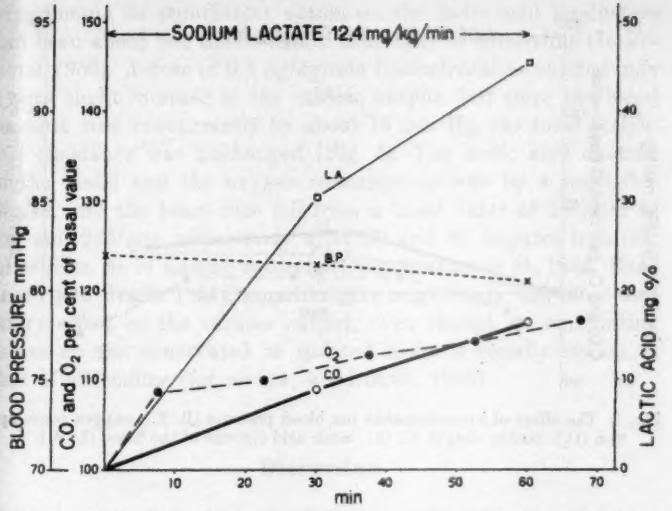


Fig. 4. The effect of sodium lactate (12.5 mg sodium lactate equivalent to 10 mg lactic acid) on: blood pressure (B. P.): oxygen consumption (O_2): cardiac output (C. O.): lactic acid content of the blood (L. A.).

pressure was almost constant throughout the infusion period, but the oxygen consumption increased by a maximum of 7 per cent. The heart rate, with a basal value of 242 beats per minute, had fallen to 212 after 30 minutes infusion, and was 214/min after 60 minutes. The respiration rate had a basal value of 63.5 per minute; it had increased to 79 after 30 minutes and to 93/min after 60 minutes.

Lactic acid infusion, like adrenaline, thus elevated the cardiac output. This effect was less than that of adrenaline, but the total circulatory picture was nevertheless fairly similar in the two series of experiments (Table V). The only major difference concerned the heart rate; here adrenaline had a slight stimulating effect but lactic acid the reverse. The decrease in heart rate may be attributed to the acidosis induced by lactic acid. BELLET, WASSERMAN and BRODY (1956) found in normal human subjects that sodium lactate had no influence on heart rate.

Sodium Lactate. The increase in the cardiac output after lactic acid infusion could be attributed both to the hydrogen and to

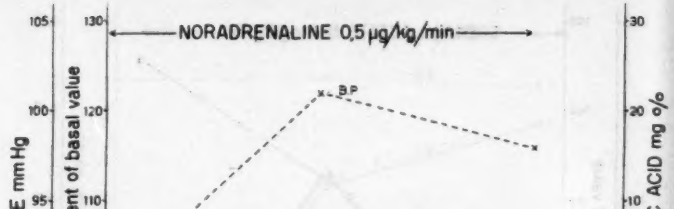


Fig. 5. The effect of l-noradrenaline on: blood pressure (B. P.): oxygen consumption (O_2): cardiac output (C. O.): lactic acid content of the blood (L. A.).

the lactate ions. NORLIN (1933), for example, in experiments on human subjects found that in acidosis induced by oral administration of ammonium chloride the cardiac output rose by 50 per cent. EULER and LILJESTRAND (1927), however, had assumed that the stimulatory effect of adrenaline on the oxygen consumption might also explain, at all events partly, the increased cardiac output. BEARN, BILLING and SHERLOCK (1951) observed in human subjects that on intravenous infusion of adrenaline the lactic acid elimination in the liver was parallel with an increased oxygen consumption and blood flow in that organ. The lactate oxidation was therefore assumed to have contributed to the rise in cardiac output.

With the aim of studying separately the response of the cardiac output to an increase in the lactate content of the blood, 10 mg/kg/min lactate in the form of racemic sodium lactate was infused in four experiments. The results are illustrated in Fig. 4. In seven of the eight determinations the cardiac output increased, the rise being almost parallel with the stimulation of the oxygen consumption. In these experiments the arterio-venous oxygen difference was almost constant, in contrast to the adrenaline and lactic acid experiments, where it fell to about 70—80 per cent of the basal value. The blood pressure showed no appreciable changes.

Noradrenaline. It seemed worth while studying the effect of noradrenaline on the cardiac output in the rabbit, for in earlier

experiments its stimulatory action on the lactic acid production had been about ten times weaker than that of adrenaline (LUNDHOLM 1950). A dose of 0.5 µg/kg/min l-noradrenaline elicited only a very slight increase in the cardiac output, but since the blood pressure rose concurrently by about 10 mm Hg, the total peripheral resistance was unchanged (Fig. 5). The lactic acid content of the blood and the oxygen consumption rose by a negligible degree, and the heart rate fell from a basal value of 236/min to 226 and 223/min respectively after 30 and 60 minutes infusion. In rabbits, as in human subjects (GOLDENBERG et al. 1948, BARCROFT and STARR 1951), noradrenaline accordingly had no stimulatory effect on the cardiac output, even though its stimulating action on the denervated or isolated heart is equally strong as that of adrenaline (for review, see EULER, 1956).

Discussion.

Causes of the Increase in Cardiac Output. The elevating effect of adrenaline on the cardiac output at an unchanged mean blood pressure may be due either to primary stimulation of the heart muscle and secondary reflex vasodilatation, or to secondary reflex stimulation of the heart muscle resulting from a primary vasodilator effect of adrenaline; or, lastly, it may be produced by a combination of primary vasodilatation and primary stimulation of the heart muscle. The practically unchanged pulse rate despite an increase in the cardiac output in my experiments and the persistent elevation of the cardiac output 10—15 minutes after the end of an intravenous infusion of adrenaline observed by GOLDENBERG et al. (1948) argued against a primary stimulating effect of adrenaline on the heart. The difference between the effects of adrenaline and noradrenaline on the cardiac output likewise indicated that the effect on the cardiac output was secondary to a reflex stimulation of the heart, which assumption was further supported by the increase of sympathetic activity by ENGELHORN (1956) after depressor doses of adrenaline. That the direct stimulating effect of adrenaline on the heart may contribute to increase the cardiac output after doses of adrenaline, which elevates the mean blood pressure is, however, beyond doubt.

Adrenaline and Noradrenaline. A substantial difference in hemodynamic effect has been demonstrated between adrenaline and

noradrenaline both in these experiments and in earlier ones conducted by GOLDENBERG et al. (1948) and BARCROFT and STARR (1951). The disparities, which are most evident in continuous intravenous infusion, can be narrowed down by studying the effect on different vascular areas: adrenaline has a pronounced dilator action on skeletal muscle vessels and hepatic vessels, but noradrenaline a much weaker effect or none at all (for review, see BARCROFT and SWAN 1953, EULER 1956). Yet on isolated vessels from those organs, as also after large doses intravenously, both adrenaline and noradrenaline have only a constrictor effect. The discrepancy in the mode of reaction to adrenaline and noradrenaline cannot, therefore, be attributed to a difference in the receptor mechanisms of the vascular smooth-muscle layer. On the other hand noradrenaline has a much weaker stimulatory effect on the lactic acid production, both qualitatively and quantitatively, than adrenaline, as has been demonstrated in the present experiments as well as earlier ones (LUNDHOLM 1950). The glycogenolytic action of noradrenaline in skeletal muscle is also much weaker than that of adrenaline (SAHYUN and WEBSTER 1933). The essential difference between the two substances that probably explains their disparate hemodynamic effects is, in my view, that the threshold concentration for adrenaline's lactic acid stimulating effect in skeletal muscle is much lower than that for its vasoconstrictor action; but with noradrenaline the corresponding concentration is the same or greater. After noradrenaline, too, a reflexogenic vasodilatation has been demonstrated in skeletal muscle (BARCROFT et al. 1954), though it is apparently insufficient to reduce the total peripheral resistance or to prevent the reflexogenic inhibition of noradrenaline's stimulating effect on the heart.

Lactic Acid Production and Carbon Dioxide Tension. In an earlier paper (LUNDHOLM 1957 a, p. 40) it was experimentally demonstrated that the lactic acid stimulating effect of adrenaline gave rise to vasodilatation in skeletal muscle in two somewhat different ways. On intra-arterial infusion, vasodilatation persisted as long as the lactic acid content of the muscle increased, and this rise continued until a fresh diffusion gradient for lactic acid had developed between the muscle and blood. With intravenous infusion of adrenaline the lactic acid content of the arterial blood rose too, inducing a progressive increase of the content in the muscle. Yet the direct cause of the vasodilatation lay in that increase of the CO_2 production which arose when the tissue bi-

carbonate neutralized the lactic acid formed. Even in experiments on intact animals, the rise in the cardiac output might accordingly be associated with an increasing CO_2 production. It is generally recognized (for review, see LUNDHOLM 1949, p. 12) that adrenaline raises the CO_2 production in intact individuals, and that this increase is largely attributable to the lactic acidosis which it induces. But since adrenaline also stimulates the respiration and diminishes the alveolar CO_2 tension (LYMAN, NICKOLLS and MCCANN 1923, ENDRES and LUCKE 1925, WHELAN and YOUNG 1953), it is *a priori* evident that the elevated CO_2 output is partly of respiratory origin and due to expiration of carbon dioxide.

After 60 minutes infusion of adrenaline, when the breathing, judging from the respiration rate, had been almost constant for 30 minutes, the CO_2 production — calculated from the arteriovenous CO_2 difference and the cardiac output — had nevertheless increased by an average of 25 per cent and the cardiac output by 39 per cent. This reflected the correlation between circulation and CO_2 production which had been demonstrated with statistical significance in the experiments on skeletal muscle (LUNDHOLM 1957 a). In the experiments with lactic acid infusion the CO_2 production rose by 50 per cent, but this increase is not comparable with that after adrenaline since an amount equivalent to that of lactic acid had already been liberated from the blood bicarbonate with the first passage through the lungs, and hence had not reached the tissues.

As discussed in an earlier paper, it would scarcely be technically possible in intact animals to duplicate exactly, by lactic acid infusion, the lactic acid producing effect of adrenaline (LUNDHOLM 1957 b). In these experiments with intravenous infusion it might only have been possible, therefore, to duplicate that increase of the lactic acid content which adrenaline produces *in the blood*, and not its primary stimulating effect on the production *in skeletal muscle*. However, an increased lactic acid content in the blood prevents the diffusion of that substance from the muscles and other tissues where it is formed. An intravenous infusion of lactic acid thus induces an increase of the tissue content of lactic acid similar to but not of the same magnitude as that caused by adrenaline.

It is unlikely, however, that the increased lactic acid content in the blood would merely induce vasodilatation by raising the CO_2 production in the tissues via an increase of lactic acid there.

EULER and LILJESTRAND (1927) assumed that the rise in the cardiac output was at least partly attributable to adrenaline's stimulating effect on the oxygen consumption. BEARN, BILLING and SHERLOCK (1951) found that the increased liver blood flow, which BRADLEY (1945) had demonstrated, was parallel with an elevated oxygen consumption and lactic acid elimination. It was therefore probable that the stimulation of the oxygen consumption which adrenaline induced by raising the lactic acid oxidation would contribute to the increase in the cardiac output.

In the experiments with sodium lactate the rises in cardiac output and oxygen consumption were parallel. The arterio-venous oxygen difference was virtually unchanged, whereas in the experiments with adrenaline and lactic acid it had been distinctly diminished. That elevation of the cardiac output which can be attributed to increased lactic acid oxidation was, in the rabbit experiments, not greater than about 10 per cent. In humans, who probably oxidize lactic acid more rapidly than do rabbits, the rise in the oxygen consumption after adrenaline is greater; and from the data reported by BEARN, BILLING and SHERLOCK (1951) it can be calculated that the elevated hepatic blood flow due to increased lactic acid oxidation should raise the cardiac output by an average of 20—25 per cent. Adrenaline's stimulating effect on the oxygen consumption reaches its maximum only after continuous infusion for some time, and persists for a while after the infusion (LUNDHOLM 1949). This probably partly explains the gradual rise in the cardiac output with continuous infusion that was observed by GOLDENBERG et al. (1948), as well as the persisting rise which they found after the infusion.

The influence of the lactic acid production on the CO_2 tension in the tissues is not, however, limited merely to increasing the CO_2 production in skeletal muscle and liver; it may also raise that tension by impairment of the CO_2 transporting capacity of the blood. To illustrate this effect Fig. 6 shows the CO_2 combining power of rabbit blood at various CO_2 tensions determined at 38° C. — In a Barcroft saturator the blood was brought into equilibrium with gas mixtures containing 20.7 per cent O_2 and varying contents of CO_2 (1.91, 4.20 and 5.93 per cent) and N_2. The upper curve shows the conditions in the blood, where the basal lactic acid content was 21.2 mg per cent; the lower curve, the corresponding state after addition of lactic acid, the content of which increased by 35.1 to 56.3 mg per cent. At higher lactic acid contents the

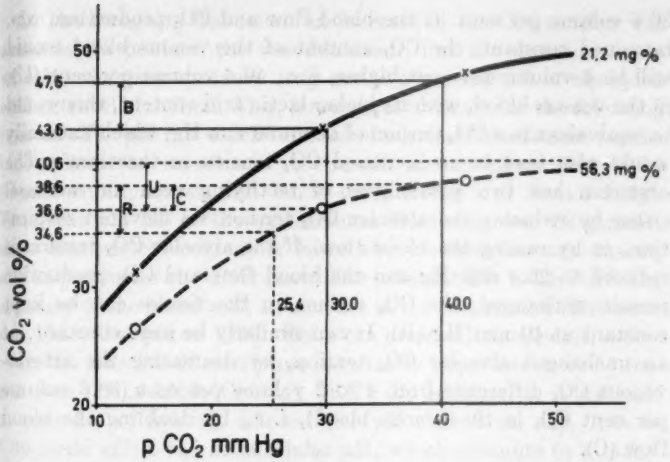


Fig. 6. The influence of lactic acid on the CO_2 transporting capacity of the blood. B: indicates the arterio-venous CO_2-difference and pCO_2 at a lactic acid content of 21.2 mg per cent. U: the arterio-venous CO_2-difference and pCO_2, if the lactic acid content of the blood had increased to 56.3 mg per cent, respiration and blood flow being unchanged. R: the arterio-venous CO_2-difference and pCO_2 if the increased CO_2 tension in the venous blood was rectified by an increased respiration. C: the same if the increased CO_2 tension was rectified by an increase in blood flow.

curve becomes flatter, as earlier pointed out by HENDERSON (1928) and LAUBENDER and MERTZ (1941). The significance of this relative to the CO_2 transporting capacity of the blood does not seem to have been observed previously, however.

Assuming the basal CO_2 tension in the arterial blood to have been about 30 mm Hg and that in the mixed venous blood to have been 40 mm Hg — which value is within the normal range of the CO_2 tension in the tissues of the rabbit (CAMPBELL 1928) — then it is evident from the upper curve that the CO_2 content of the arterial blood was 43.6 volume per cent and of the venous blood 47.6 volume per cent, the arterio-venous CO_2 difference being 4 volume per cent (B in Fig. 6.) These figures are representative of the blood CO_2 values that were recorded under basal conditions in the rabbit at determination of the cardiac output. It will be seen from Fig. 6 that if the lactic acid content rose to 56.3 mg per cent and the alveolar CO_2 tension remained constant at 30 mm Hg, the CO_2 content of the arterial blood should decrease to

36.6 volume per cent. If the blood flow and CO_2 production, too, remained constant, the CO_2 content of the venous blood would still be 4 volume per cent higher, *i. e.*, 40.6 volume per cent (U); in the venous blood, with its higher lactic acid content, this would be equivalent to a CO_2 tension of about 60 mm Hg, which naturally would also lead to an increased CO_2 tension in the tissues. The organism has two possibilities of rectifying such an increase: either by reducing the alveolar CO_2 tension via elevated respiration, or by raising the blood flow. If the alveolar CO_2 tension is reduced to 25.4 mm Hg and the blood flow and CO_2 production remain unchanged, the CO_2 tension in the tissues can be kept constant at 40 mm Hg (R). It can similarly be kept constant, at an unchanged alveolar CO_2 tension, by decreasing the arteriovenous CO_2 difference from 4 to 2 volume per cent (38.6 volume per cent CO_2 in the venous blood), *i. e.*, by doubling the blood flow (C).

It has already been mentioned that the alveolar CO_2 tension falls after adrenaline; and the same is true after hard muscular exercise with an increased lactic acid content in the blood (COMROE 1944, BANNISTER, CUNNINGHAM and DOUGLAS 1954) or following administration of ammonium chloride (HALDANE et al. 1928, NIELSEN 1936). Numerous investigators have regarded this as evidence against a regulatory significance of the CO_2 tension. As will be evident from this discussion, a fall of the CO_2 tension in the arterial blood may well be caused by a rise of that in the tissues; so that this should be taken, rather, as evidence of its significance.

LANGE et al. (1950) found that intravenous infusion of gluconic acid or acid sodium phosphate in dogs under artificial respiration caused a pronounced fall of the peripheral resistance at an unchanged mean blood pressure and increased cardiac output. The experiments on the rabbit indicated that this animal largely rectified the diminished CO_2 transporting capacity of the blood by increased respiration. After 60 minutes lactic acid infusion the cardiac output had thus risen only by an amount that could be attributed to the increased lactic acid oxidation, whereas the respiration rate had risen from 63.5 to 93 per minute. Experiments on man (BARCROFT and COBBOLD 1956) and cat (LUNDHOLM 1957 b) indicated however that in these species the diminished CO_2 transporting capacity of the blood caused by an increase of the lactic acid content was also rectified by vasodilatation and an increase of the blood flow.

In the foregoing discussion an increase of the CO_2 tension was assumed to produce the vasodilatation, but the question arises whether the primary cause may not be a lowered pH of the blood and extracellular fluid. This, however, is unlikely for the following reasons. The pH fall which infusion of adrenaline causes in venous blood from the muscles is very small, amounting to 0.02—0.05 (GOLLWITZER, MEIER and FACK 1949, HILLE and FESKE 1956). True, the pH fall in the extracellular fluid may well be greater, since the protein content is lower and erythrocytes are absent; and in particular the latter contribute substantially to the buffering capacity of the blood with an increase in the CO_2 tension (HENDERSON 1928, ROUGHTON 1935). From the practical standpoint, moreover, the muscle cell membrane is virtually impermeable to hydrogen ions (HILL 1955, CONWAY 1957). In addition, it is difficult to conceive of how an extracellular pH shift from, say, 7.35 to 7.30 could affect the intracellular pH, which amounts to a maximum of 6.9, since Donnan equilibrium occurs very slowly (CONWAY 1957). Even small pH shifts in the tissues will be associated, however, with substantial changes in the CO_2 tension, and since carbon dioxide freely penetrates the cell membrane it is probable that extracellular pH alterations influence, via a rise of the CO_2 tension in the extracellular fluid, the intracellular CO_2 tension.

Lastly, a few words about the physiologic significance of adrenaline's stimulatory effect on the lactic acid production. In the opinion of CANNON (1929) the lactic acid producing and the glycogenolytic effects of adrenaline in skeletal muscle merely constituted a pharmacologic action of no consequence physiologically. If the stimulation by adrenaline of the lactic acid production is the primary cause of the vasodilatation in skeletal muscle, then the former effect however should be regarded as a stage of the emergency reaction which, according to CANNON, adrenaline induces. Of interest in this connection is an observation made by EULER and FOLKOW (1953), in cats; namely, that a pain stimulus — which would conceivably elicit a flight or defense reaction — was associated with a higher percentual content of adrenaline in venous blood from the adrenals than was a stimulus that led to blood pressure regulation, in which case noradrenaline was the predominant hormone. CELANDER (1954) found that the most pronounced circulatory response to stimulation of the adrenals lay in vasodilatation in skeletal muscle. If the organism, by secretion of adrenaline and via stimulation of the lactic acid production in

the muscles, can raise the blood flow in skeletal muscle, and if this is one phase of an emergency reaction, then the significance of the lactic acid production during muscular exercise will have to be viewed in a different light. In moderately heavy muscular work the lactic acid production in the muscles appears to be limited mainly to the initial stage of exercise (BANG 1936, FLOCK, INGLE and BOLLMAN 1939); and it has been assumed that the production continues until the blood flow is adequate, so that the muscles can cover their energy requirements fully by oxidative processes. Since lactic acid also possesses a vasodilator effect it is probable that the muscles, in their ability to form lactic acid, have a mechanism that not only provides energy during an inadequate supply of oxygen but at the same time establishes conditions for an adequate supply of oxygen.

Summary.

1. The effects of adrenaline, noradrenaline, lactic acid and sodium lactate, in intravenous infusion, upon the cardiac output, blood pressure, oxygen consumption, heart and respiration rates, and lactic acid content of the blood were investigated in unanesthetized rabbits. Control experiments were performed with infusion of saline alone.

2. Both adrenaline and lactic acid infusions were followed by a rise in the cardiac output but no change in blood pressure, which finding indicated a reduction of the total peripheral resistance. The increase in cardiac output was greater than the rise in oxygen consumption.

3. After sodium lactate the cardiac output rose only to the same degree as the oxygen consumption.

4. Noradrenaline elevated the cardiac output, oxygen consumption and lactic acid production very slightly, concurrently with a pressor effect.

5. The stimulating action of adrenaline in a low concentration on the cardiac output is thought to be largely of reflex origin, resulting from a primary vasodilator effect caused by adrenaline's stimulation of the lactic acid production in skeletal muscle. Lactic acid induces vasodilatation in the following ways: (1) Through the rise in the lactic acid content of skeletal muscle, CO_2 is liberated from the tissue bicarbonate and vasodilatation is produced via the increased CO_2 tension. (2) When the lactic acid content

rises in the blood, the latter's CO_2 transporting capacity is impaired, and this also tends to raise the CO_2 tension in the tissues. (3) With oxidation of the lactate the blood flow increases, chiefly in the liver.

6. The discrepancies in the hemodynamic effects of adrenaline and noradrenaline are attributed to the fact that the former hormone, even in low concentrations, has a powerful stimulatory action on the lactic acid production, but the latter almost none.

7. The lactic acid producing effect of adrenaline in muscle is assumed to be a phase of the mechanism by which it elicits an emergency reaction in the organism.

References.

Allen, W. J., H. Barcroft and O. G. Edholm, J. Physiol. (London.) 1946. *105*. 255.

Bang, O., Skand. Arch. Physiol. 1936. *74*. Suppl. 1051.

Bannister, R. G., D. J. C. Cunningham and C. G. Douglas, J. Physiol. (London.) 1954. *125*. 90.

Barcroft, H. and A. F. Cobbold, J. Physiol. (London.) 1956. *132*. 372.

Barcroft, H., P. Gaskell, J. T. Shepherd and R. F. Whelan, J. Physiol. (London.) 1954. *123*. 443.

Barcroft, H. and I. Starr, Clin. Sci. 1951. *10*. 295.

Barcroft, H. and H. J. C. Swan, Sympathetic Control of Human Blood Vessels, Arnold and Co. London 1953.

Barnett, A. J., R. B. Blacket, A. E. Depoorter, P. H. Sanderson and G. M. Wilson, Clin. Sci. 1950. *9*. 151.

Bearn, A. G., B. Billing and S. Sherlock, J. Physiol. (London.) 1951. *115*. 430.

Bellet, S., F. Wasserman and J. I. Brody, Amer. med. Sci. 1956. *231*. 274.

Bradley, St. E. J., Clin. Invest. 1946. *25*. 918.

Bülbring, E. and J. H. Burn, J. Physiol. (London.) 1942. *101*. 289.

Campell, J. A., J. Physiol. (London.) 1928. *65*. 255.

Cannon, W. B., Physiol. Rev. 1929. *9*. 399.

Celander, O., Acta physiol. scand. 1954. *32*. 116.

Comroe, J. H. Jr., Physiol. Rev. 1944. *24*. 319.

Conway, E. J., Physiol. Rev. 1957. *37*. 84.

Cori, C. F., G. T. Cori and K. W. Buchwald, Amer. J. Physiol. 1930. *93*. 273.

Dale, H. H. and A. N. Richards, J. Physiol. (London.) 1927. *63*. 201.

Dörner, J., Pflügers Arch. ges. Physiol. 1953. *257*. 464.

Dörner, J., Pflügers Arch. ges. Physiol. 1956. *262*. 265.

Endres, G. and H. Lucke, Z. ges. exp. Med. 1925. *45*. 89.

Engelhorn, R., Arch. exp. Path. Pharmak. 1957. *231*. 219.

Euler, U. S. v., J. Physiol. (London.) 1938. *92*. 111.

Euler, U. S. v., Noradrenaline, Thomas, Springfield. 1956.

EULER, U. S. v. and B. FOLKOW, Arch. exp. Path. Pharmak. 1953. *219*. 242.
EULER, U. S. v. and G. LILJESTRAND, Skand. Arch. Physiol. 1927. *25*. 243.
FLOCK, E. V., D. J. INGLE and J. L. BOLLMAN, J. biol. Chem. 1939. *129*. 99.
FRIEDEMANN, TH. E. and J. B. GRAESER, J. biol. Chem. 1933. *100*. 231.
GERNANDT, B., G. LILJESTRAND and Y. ZOTTERMAN, Acta physiol. scand. 1946. *11*. 301.
GIRLING, F., Amer. J. Physiol. 1951. *164*. 400.
GOLDENBERG, M., K. L. PINES, E. DE F. BALDWIN, D. G. GREENE and C. E. ROH, Amer. J. Med. 1948. *5*. 792.
GOLLWITZER-MEIER, KL. and H. FACK, Pflügers Arch. ges. Physiol. 1949. *251*. 344.
GRUHZIT, C. C., W. A. FREYBURGER and G. K. MOE, J. Pharmacol. 1954. *112*. 139.
HALDANE, J. B. S., G. C. LINDER, R. HILTON and F. R. FRASER, J. Physiol. (London.) 1928. *65*. 412.
HARVEY, ST. C. and M. NICKERSON, J. Pharmacol. 1952. *108*. 281.
HENDERSON, L. J., Blood. London. 1928.
HEYMANS, C., J. J. BOUCKAERT and M. WIERZUCKOWSKI, Arch. int. Pharmacodyn. 1957. *156*. 37.
HEYMANS, C. and G. VAN DEN HEUVEL-HEYMANS, Arch. int. Pharmacodyn. 1950. *83*. 520.
HILL, A. V., Proc. Roy. Soc. B. 1955. *144*. 1.
HILLE, H. and H. J. FESKE, Pflügers Arch. ges. Physiol. 1949. *251*. 344.
LANGE, K., F. GRAIG, D. WEINER and V. TCHERTHOFF, Fed. Proc. 1950. *9*. 294.
LAUBENDER, W. and B. MERTZ, Arch. exp. Path. Pharmak. 1941. *198*. 12.
LUNDBERG, A., Acta physiol. scand. 1952. *26*. 252.
LUNDHOLM, L., Acta physiol. scand. 1949. *19*. Suppl. 67.
LUNDHOLM, L., Acta physiol. scand. 1950. *21*. 195.
LUNDHOLM, L., Acta physiol. scand. 1957 a. *39*. Suppl. 133.
LUNDHOLM, L., Acta physiol. scand. 1957 b. *40*. 344.
LYMAN, R. S., E. NICHOLLS and W. S. MCCANN, J. Pharmacol. 1923. *21*. 343.
MALMÉJAC, J. J., J. Physiol. (London.) 1955. *130*. 497.
MARRAZZI, A., Amer. J. Physiol. 1939. *127*. 738.
NIELSEN, M., Skand. Arch. Physiol. 1936. *74*. Suppl. 10.
NORLIN, G., Skand. Arch. Physiol. 1933. *67*. 170.
PETERS, J. P. and D. VAN SLYKE, Quantitative Clinical Chemistry. *2*. London. 1932.
ROUGHTON, F. J. W., Physiol. Rev. 1935. *15*. 241.
SAHYUN, M. and G. E. WEBSTER, Arch. int. Pharmacodyn. 1933. *45*. 291.
SWAN, H. J. C., J. Physiol. (London). 1951. *112*. 426.
WHELAN, R. F., J. Physiol. (London.) 1952. *118*. 575.
WHELAN, R. F. and I. M. YOUNG, Brit. J. Pharmacol. 1953. *8*. 98.

Acta physiol. scand. 1958. 43. 51—70.

From the Department of Histology, University of Göteborg and Fibiger Laboratoriet, University of Copenhagen.

Changes in the Content of Nucleic Acids in Organs of Young Rats During Arrested Growth.

By

SØREN LØVTRUP and VIRGINIA L. SWANSON.[1]

Received 1 March 1958.

Introduction.

Much attention has been paid to the quantitative changes occurring in the organs and tissues of young rats, with growth arrested in various ways, *e. g.* underfeeding, deficiency of proteins, vitamins, salts, etc. These studies have mainly been confined to the determination of changes in weight (W). We have extended this work by measuring the content of total nitrogen (TN), desoxyribonucleic acid (DNA), and ribonucleic acid (RNA) in organs of young rats arrested in growth by protein deficiency. On the assumption that the DNA content per cell nucleus is constant, the DNA determinations ought to give information about the changes in the number of cells during starvation. Special interest was focussed on the question of the possible irreversible effects of growth arrest. Our material therefore includes animals, which after two months of growth arrest were fed *ad libitum*.

[1] Work done during tenure of U.S.P.H.S. Research Fellowship. Present address: Laboratory of Pathology, Yale University School of Medicine, New Haven, Connecticut.

Material and Methods.

Biological Material. 128 female weanling rats[1] were divided into six groups (1—6) as shown in Table I. Female rats were used exclusively because they are more resistant to the deleterious effects of inadequate dietary protein (JACKSON 1936). They were 24—25 days old and weighed approximately 50 g at the start of the experiment.

Subsequently, another group of 71 female weanling rats of approximately the same age and weight were divided into six groups (7—12 in Table I).

Of the 172 animals that survived, 129 provided material for chemical determinations while some organs of 43 rats were examined histologically.

Diet. Each experimental animal was quartered in its own cage and fed a diet similar to that used by LIMSON and JACKSON (1932), which is a mixture of 75 g of sucrose, 4.5 g of salts (MacCollum's Mixture) and 20 g of lard. This portion of the diet was given *ad libitum*. In addition, 1—2 drops of cod liver oil were administered orally each day by hand. A mixture of equal parts of yeast and wheat germ was made up separately, and an amount (varying between 0.5 and 3 g a day) sufficient to maintain body weight at 50 g was scattered as a dry powder over the top of the sugar-lard-salt mixture. Tap water was supplied in bottles to each cage. Experimental animals were weighed every second day.

Control rats were kept in larger cages in the same room. Two or three animals occupied a single cage. They ate the laboratory stock diet of ground wheat, ground maize, lucerne flour, fish flour, skin milk powder, cod liver oil and vitamin E oil. Control rats were weighed once a week.

Autopsy procedure. Each animal was anesthetized with ether, and the heart removed immediately. The liver was perfused *in situ* with saline to wash out as much blood as possible. The remaining organs (lungs, thymus, heart, spleen, kidneys, and brain) were removed, stripped clean of fat and fascia where present, rinsed in saline and placed in containers in an ice bath. They were weighed immediately and placed in 10 per cent formalin. Livers were incised in several places so that fixation could take place more rapidly. After fixation the organs were kept in alcohol until the chemical determinations were to be made.

Observations on appearance of animals. All control animals remained healthy and grew normally.

The experimental animals ate well, were active and showed no evidence of vitamin deficiencies at any time. Even though body weight was maintained within 5 g of the original 50 g weight, the rats appeared to become progressively more emaciated which can probably be ascribed

[1] Subline of Wistar strain obtained from Løvens Kemiske Fabrik, Copenhagen.

Table I.

Survey of the number of animals and survivors in the various experimental groups, and the treatment to which the animals in the groups were subjected.

Group	Treatment	No. of Animals		No. of Survivors	
		Chemical Examination	Histological Examination	Chemical Examination	Histological Examination
1	Killed at beginning of experiment	15	5	(15)	(5)
2	Protein-free diet for 2 months	16	4	12	3
3	Stock diet for 2 months	15	5	15	5
4	Protein-free diet for 4 months	21	8	8	4
5	Stock diet for 4 months	14	5	14	5
6	Protein-free diet for 2 months followed by stock for diet 2 months	15	5	12	4
	Totals	96	32	76	26
7	Killed at beginning of experiment	9	4	(9)	(4)
8	Protein-free diet for 1 month	10	4	10	4
9	Stock diet for 1 month	7	2	7	2
10	Protein-free diet for 2 months followed by stock diet for 6 months	10	5	9	5
11	Stock diet for 8 months	8	2	8	2
12	Protein-free diet for 10 days	10	0	10	0
	Totals	54	17	53	17

to some skeletal growth, although no actual measurements were made (cf. LIMSON and JACKSON 1932).

Twenty-seven dieting rats died during the experimental period. For 12—16 hours before death they were markedly lethargic, huddled in the corner of the cage, shivered and did not eat. Two were obviously jaundiced. Post-mortem examinations revealed acute diffuse necrosis of the liver in all cases, but no gross or microscopic abnormalities of any other tissue were observed. These animals succumbed after 36 to 110 days on the protein-free diet; the average period of survival was 67 days. The dieted animals that survived had no gross or histological lesions of the liver. Further histological findings are discussed below.

Since vitamin E deficiency combined with certain amino acid deficiencies has been shown to result in acute hemorrhagic necrosis of the liver in rats, a vitamin E determination was carried out on the wheat germ supplied in the protein-free diet. It contained 0.25 mg per g so that the dieted animals received amounts varying between 125—750 μg per day, an amount well within the range of the normal daily vitamin E requirement for the prevention of deficiency symptoms in rats (MASON 1940). It is possible that deficiencies of amino acids, particularly cystine, or of unsaturated fatty acids, or both, were the cause of the observed liver necrosis.

Homogenization. Very efficient homogenization was necessary because the organs had been hardened during the time they were kept in alcohol. As the homogenization volumes necessarily had to be kept down to a range between 10 and 25 ml, a special microhomogenizer was constructed. The apparatus consisted of an adapter fitted on a drilling machine, rotating at 24,000 rpm. A small blade with six cutting edges was screwed on the shaft of the drill, and a 50 ml centrifuge tube clamped on the adapter.

Determination of TN. The determinations were made according to the ordinary Parnas-Wagner micro-Kjeldahl method on appropriate dilutions of the homogenates.

Determination of RNA and DNA. The method employed was a modification of the Schneider procedure. As the tissue samples had been stored in alcohol before the chemical determinations were performed, no extraction was made with alcohol-ether. Procedure: 2 ml homogenate (about 10 per cent, based on the wet weight) was extracted with 5 ml 12 per cent cold trichloracetic acid (TCA), centrifuged and extracted again with 5 ml cold TCA. After centrifugation 4 ml 6 per cent TCA was added, the tubes heated for 15 minutes in a boiling water bath, centrifuged, and again extracted with 4 ml hot TCA, this time for 5 minutes. The two extracts were poured into a 10 ml volumetric flask and the volume subsequently filled up to the mark.

The dilution of the extracts before photometric determination varied somewhat, depending upon the nucleic acid content of the particular tissue. For several tissues (*e. g.* liver) the following procedure was found expedient. DNA: 1 ml undiluted extract or standard solution (400 μg DNA per ml) + 2 ml diphenylamine reagent (10 mg diphenylamine (Merck) and 27.5 μl concentrated H_2SO_4 per ml glacial acetic acid) was heated for 10 min in boiling water. Absorbance was read at 600 mμ. RNA: 200 μl undiluted extract or standard solution (400 μg RNA per ml) + 4 ml orcinol reagent (5 mg orcinol (Merck) and 0.5 mg $FeCl_3$ per ml concentrated HCl) was heated for 20 min in boiling water. Absorbance was read at 670 mμ. The RNA values were corrected for DNA by an empirical correction factor. The nucleic acid content of commercial standard preparations was calculated from the P content. Each ho-

mogenate was extracted twice, and on each extract two determinations of each nucleic acid was made. The values obtained are thus averages of four determinations.

Results.

Body weights.

The growth curves seen in Fig. 1 show normal weight gains for animals fed the stock diet (groups 3, 5, 9 and 11). It was possible to maintain body weight at approximately 50 g in groups 2, 4, 6, 8, 10 and 12 by feeding a protein-free diet for between 10 days and four months (group 12 is not represented in Fig. 1).

Animals fed the stock diet following two months of the protein-free diet (6 and 10) showed weight gains very similar to those of the stock-fed controls during the period of most rapid growth.

Histological findings.

In the animals dying of liver necrosis, two types of early alteration of liver cells were seen. These two distinct types have recently been described by FITE (1954) in association with acute nutritional necrosis of the liver in rats. The first consists of karyorrhexis and karyolysis of the nucleus which precedes any visible alteration of the cytoplasm. In the second type, small granules and clumps of eosinophilic refractile material appear in the cytoplasm as the primary change followed by a gradual disappearance of the nucleus. Both processes can proceed to frank necrosis. In the animals dying of liver necrosis in these experiments, both degenerative processes were sometimes found in the same liver. The necrosis had no definite pattern of distribution. Hepatic cells adjacent to portal triads were intact in many instances, although not always. Marked sinusoidal congestion around necrotic areas was observed but no true hemorrhages occurred except in areas of complete disintegration of liver architecture.

In the last three animals that died, on the 101st, 108th, and 110th days, foci of regenerating liver cells were present, suggesting perhaps a prolongation of the process of necrosis. No regeneration was seen in animals that succumbed earlier than the 101st day.

As FITE (1954) has emphasized, there is a normal histological appearance of the liver almost up to the point at which necrosis develops. None of the livers of our surviving protein-starved

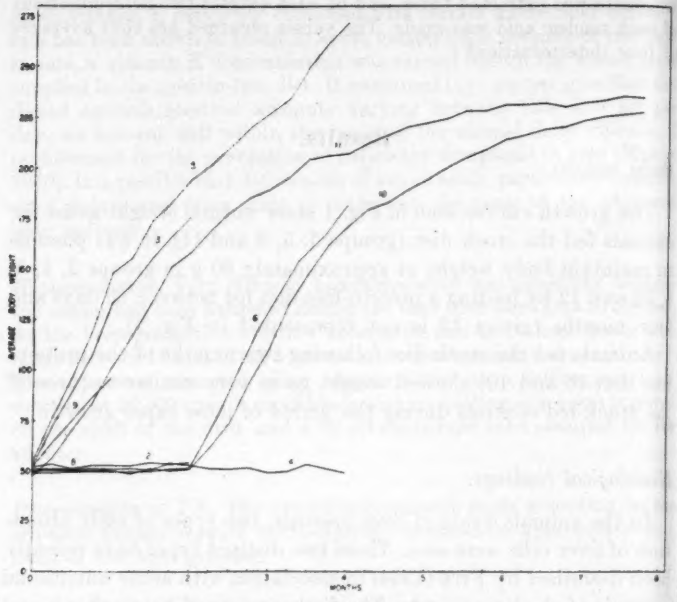


Fig. 1. Growth curves for the animals in the various control and experimental groups (except group 12).

animals showed any evidence of regeneration or scarring. This does not necessarily mean that no attacks of nonfatal necrosis appeared in the group; it may indicate that recovery of the liver is complete and that there is no permanent residual fibrosis. However, the rats on the protein-free diet were watched closely and none ever developed noticeable symptoms without dying within 36 hours. It is important that all animals used for chemical analysis were free of symptoms at the time they were killed.

The hepatic cells of protein-starved animals were thought to be smaller and actual counts of nuclei contained in a ruled square inserted into the eyepiece revealed this to be true (Table II). This increase in the number of nuclei during the period of protein deficiency is, as will be shown, not associated with any increase in the content of DNA.

Examination of the hearts and lungs of all groups revealed no histological abnormality. The thymus and spleen from control,

Table II.

The results of counting nuclei in livers of control, starved and refed animals.

Group	Squares counted	Average number of nuclei per square	Average liver weight
1	80	55	2.72
2	120	98	3.10
3	200	55	8.22
4	160	116	2.49
5	160	52	7.82
6	160	65	7.18

protein-starved and refed groups were alike in appearance. There was no noticeable diminution in their lymphocyte content as has been shown to occur during short periods of total starvation (SALKIND 1915, HELLMAN 1926). The tubular epithelial cells from the kidneys of animals subjected to protein deprivation appear smaller than those of control groups; meanwhile, as with the liver, the average kidney weight has remained constant or fallen slightly. Further, tubular crystalline casts localized near the corticomedullary junction were seen in the protein-starved groups. Similar casts have been reported in association with dietary necrotic liver degeneration in rats. However, the livers of our animals were normal in appearance.

Some of the brains revealed pyknosis of cortical ganglion cells and of nerve cells of the basal ganglia, but since two control animals also showed these changes, they were believed to be caused by trauma inflicted during removal of the brains. An incidental finding in the protein-starved animals was a considerable thinning of the walls of the small intestine which was otherwise normal in appearance.

Chemical determinations.

Liver. The most comprehensive results were obtained on the liver because each liver was weighed and analyzed separately. For technical reasons one liver in group 1, and three in group 7 had to be excluded. The results are compiled in Table III, a closer study of which reveals the considerable variation of weight and chemical

Table III.

The results of weight and chemical determinations on individual livers. The numbers in the upper row represent the mean and the standard error of the mean, and in the bottom row is given the minimum and maximum values observed.

Group	n	W (g)	TN (mg)	DNA (mg)	RNA (mg)
1	14	2.72±0.13 2.11—4.08	61±2 53—80	9.1 ±0.5 7.10—13.70	19.5±1.1 12.4—27.2
2	12	3.10±0.10 2.42—3.65	56±2 45—72	10.0±0.5 5.79—11.70	17.3±1.6 10.1—25.2
3	15	8.22±0.21 6.31—9.30	200±7 145—249	21.0±0.9 17.1—26.2	49.3±2.3 33.3—66.3
4	8	2.49±0.10 2.18—2.89	55±2 46—60	9.9±0.4 9.2—12.2	16.1±1.3 9.0—21.0
5	14	7.82±0.24 6.11—9.47	216±6 176—251	24.8±0.9 20.3—33.0	53.5±2.8 37.0—77.5
6	12	7.18±0.33 5.28—10.19	195±7 142—238	21.0±1.4 13.5—31.0	43.6±2.3 32.8—60.3
7	6	2.81±0.15 2.36—3.19	60±8 33—95	10.4±1.8 4.7—16.1	16.2±2.2 10.5—24.6
8	10	3.43±0.18 2.62—4.13	53±4 28—66	8.4±0.4 6.9—10.8	15.3±0.8 9.1—18.3
9	7	6.96±0.52 4.76—8.61	110±11 69—167	13.4±1.0 11.3—17.4	32.4±2.4 24.5—49.3
10	8	7.89±0.55 5.85—10.30	200±14 139—245	27.1±1.3 20.1—31.4	50.8±3.8 39.0—67.0
11	10	7.74±0.18 6.90—8.54	200±8 163—253	25.2±0.7 23.1—30.0	49.9±1.4 40.5—54.0
12	10	2.82±0.08 2.46—3.19	43±2 37—57	8.0±0.2 7.0—9.0	13.7±0.4 11.4—15.3

composition in livers from different rats. In Fig. 2 the relative changes have been plotted against time. The liver weight in the controls is seen to increase about 2.8 times in the first two months, but after this time no significant changes occur. Protein deficiency for four months is seen to cause only a very slight decrease in W. When stock diet is supplied after two months of starvation the weight is normal two months later. Essentially the same picture is seen in the other three diagrams, except that for TN the maximum relative increase is higher ($\sim$ 3.5), and an apparently significant loss occurs during the first 10 days. For RNA and DNA the final relative increase is about 2.8, and DNA is seen to increase for four months. The curves indicate slight losses during the period of

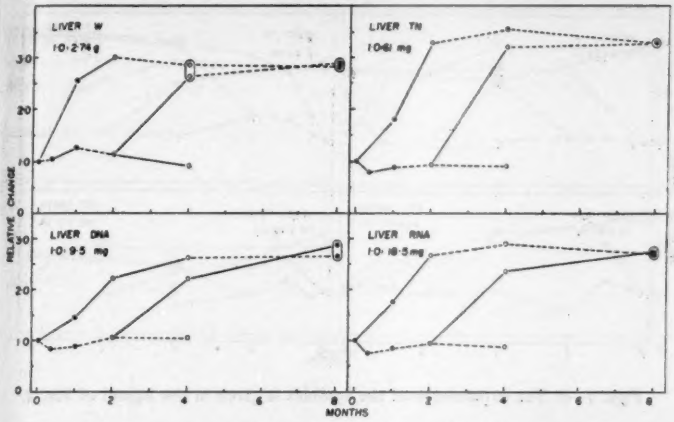
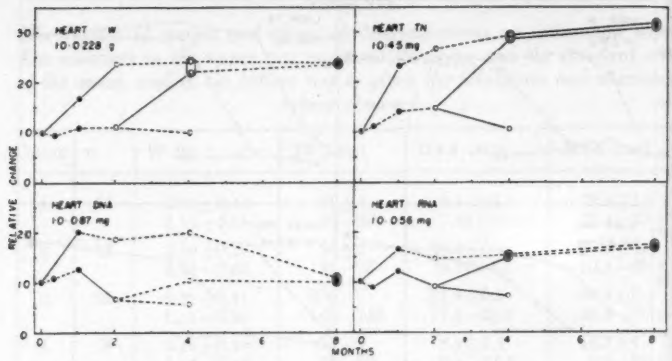


Fig. 2. The open and filled circles represent the two batches of animals. The difference of the means for the first point in the two batches of animals (groups 1 and 7) was tested statistically for all 4 quantities for the five organs where this was possible (liver, heart, kidney, brain and lung). Only for kidney weight and brain RNA were significant differences observed ($p > 0.05$). It was therefore decided to unite these two groups for these five organs, whereas in spleen and thymus each batch was calculated separately. A stippled line connecting two points at different times indicate that the difference between the values is statistically insignificant ($p < 0.05$). For points at the same time an insignificant difference is indicated by a frame around the points.

starvation, but only for RNA was a significant loss observed. After starvation, six months of stock diet was necessary to give normal DNA and RNA values.

Heart. The hearts were analyzed in groups of 4—6 (except one group containing only 2). The results are shown in Fig. 3. It is seen that in the controls the weight more than doubles during the first two months, after which no further increase is observed. During starvation the weight remains constant, and after starvation normal weight is reached in two months. For TN a certain increase is observed during the whole experiment in the controls. During starvation, TN first increases slightly, then it falls to about the original value. Normal TN content is reached two months after refeeding. In the controls the DNA content increases during the first month, remains constant for the next three months, and decreases again during the last four months. During starvation DNA first increases, subsequently decreases, at first quite fast, later more slowly. Stock diet following starvation permits a slight



Figs. 3—8. The explanation of the symbols is given in the legend of Fig. 2.

increase in DNA, but the DNA content of the controls is reached only because of the loss of DNA in the latter. For RNA the increase lasts one month, after which the RNA remains constant for seven months. The changes during starvation are similar to those of DNA, and it is seen that normal RNA values are reached in 2 months in the starved animals.

Kidney. The results on kidneys are shown in Fig. 4. It is seen that in the controls the weight increases rapidly during the first month and subsequently more slowly to about 2.4 times the original weight. During protein deficiency kidney W remains nearly constant. After six months of stock diet the weight of the controls has been reached. Essentially the same is shown by the TN results, only the relative increase in the controls is higher ($\sim$ 2.7), and there may be a slightly greater loss during starvation. Contrary to W and TN, the DNA content does not change after the first month. During starvation, a certain loss probably occurs. In the animals starved for two months DNA increases when normal diet is supplied, but after two months the DNA content is still not significantly different from the animals kept four months on the protein-free diet. After another four months, however, the DNA content has reached that of the controls. The RNA content increases considerably during the first month, and after this a slight, but con-

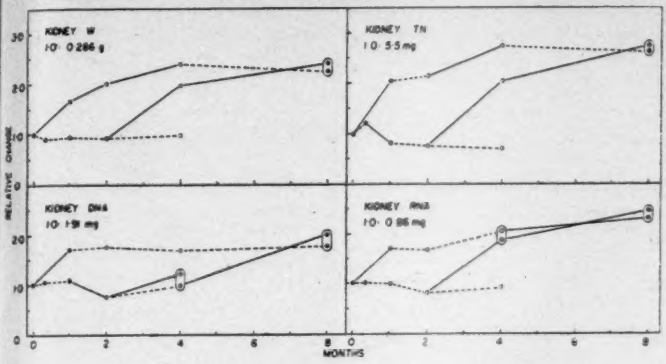


Fig. 4.

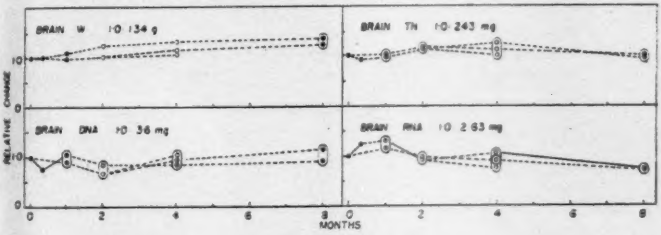


Fig. 5.

tinuous increase is observed. Following starvation, the normal content is reached already after two months, otherwise the results are very much like those seen for DNA.

Brain. For brain weight a slight tendency to increase is observed in the controls (Fig. 5), and this increase is significantly retarded during starvation, but the normal value is reached after six months of normal diet. For TN, DNA, and RNA no significant changes are observed.

Lung. For lung the results are very similar to those obtained with liver, especially with respect to W and TN, with the one exception that the relative increase is slightly lower for lung (Fig. 6). The DNA content of the lungs, on the other hand, is seen to increase

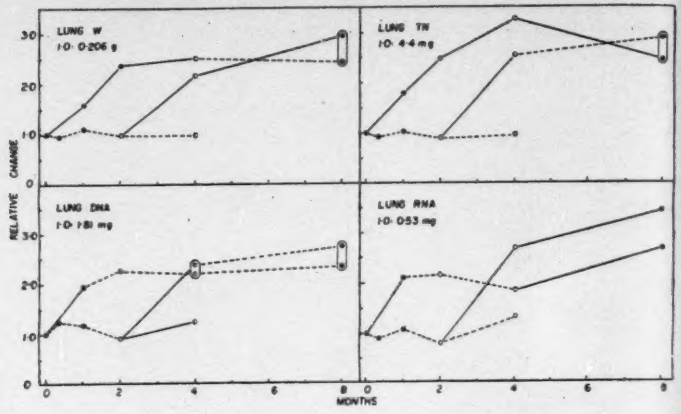


Fig. 6.

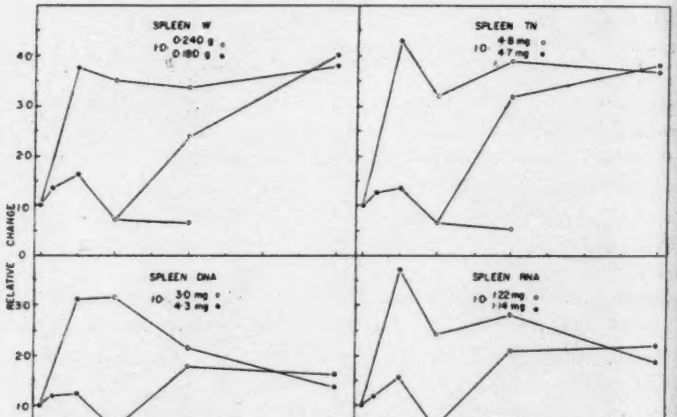


Fig. 7.

only for 1—2 months in the controls. After two months protein starvation, a stock diet for two months is sufficient to permit complete restoration of the DNA content. The results for RNA are peculiar, showing increase during the first month, constancy

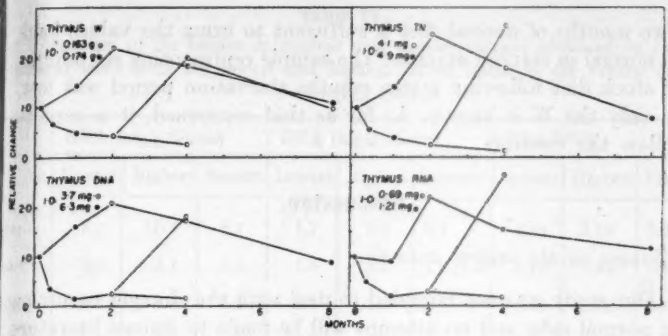


Fig. 8.

during the next three months, and a further increase during the last four months. In the starved animals RNA is significantly higher after two months of protein diet, and this difference is preserved for the rest of the experiment.

Spleen. Spleen and thymus from all animals in one experimental group were each analyzed in one pool. From Fig. 7 it is seen that spleen W increases considerably (3.5 times) during the first month, and remains at this level throughout the eexprimental period. Essentially the same is seen for TN in the controls, whereas both DNA and RNA show definite decreases during the last 6—7 months of the experimental period. During starvation all four curves agree in showing increases during the first month, considerable decreases during the second, and constancy or slight decreases during the last two months of starvation. For all four quantities the final levels lie around 50 per cent of the initial values. Feeding of normal diet to starved animals leads to normal values, at least after six months.

Thymus. In the controls increases in all four quantities are seen during the first two months. After this time they all decrease, reaching at the end of eight months levels not far from the original ones (Fig. 8). During starvation all substances, and particularly the nucleic acids, suffer substantial decreases. Already after one month the DNA and RNA contents lie at about 15 per cent of the original, remaining there during the whole experimental period.

Two months of normal diet is sufficient to bring the values back to normal in starved animals. The sample representing six months of stock diet following a two months starvation period was lost, so only the W is known. As far as that concerned, it is seen to follow the controls.

Discussion.

Changes in the control animals.

Our study was not intended to deal with the changes occurring in normal rats, and no attempt will be made to discuss literature dealing with the changes in weight and TN. It should only be mentioned that Andreasen (1943), working with the same strain as we, has published recordings of the changes in weight for a number of organs, including all those investigated by us except brain. Our results are, to the extent that comparison may be made, consistent with his except for minor details. As far as the TN values are concerned, it will be noticed that on the whole they run quite parallel with the W values but that they, as a general rule, show higher relative increases than the latter.

Very little seems to be published about the changes in the nucleic acids in rat organs during growth. The data for liver by Fukuda and Shibatani (1953) cannot be used for comparison as they were obtained on male rats, and the weight results by Andreasen indicate that the male livers grow for a longer time and reach higher end values than female ones.

To compare our results on nucleic acid determinations on adult rat tissues the data compiled in the review by Leslie (1955) may be used. These values vary considerably, and some of this variation is, as discussed by Leslie, due to the methods employed. In Table IV we have given the values obtained by us (group 11) together with the lowest and highest values from Leslie, without regard to the methods used. It will be noticed that, except for spleen, our RNA values lie between (or close to (brain)) the limits. Our DNA values are above the highest value for liver, kidney and brain, and the corresponding RNA/DNA ratios are lower. For thymus our DNA value is lower than those listed by Leslie, but it is obvious from Fig. 8 that this value is very much dependent upon the time at which the analysis is made. The value for heart DNA in parenthesis corresponds to the maximum level (Fig. 3). The

Table IV.

Comparison of the values of nucleic acid determinations obtained in the present work with the lowest and highest values listed in the review by LESTIE *(1955).*

	RNA (mg/g tissue)			DNA (mg/g tissue)			RNA/DNA		
	Lowest	Highest	Present	Lowest	Highest	Present	Lowest	Highest	Present
Liver ...	7.7	10.2	6.7	1.7	2.4	3.5	3.28	5.15	1.91
Heart ..	1.2	3.1	1.7	1.5	3.1	1.8 (3.2)	0.85	1.03	0.94 (0.53)
Kidney .	2.9	7.0	3.1	2.7	4.2	5.8	0.89	2.46	0.53
Brain ..	1.4	3.7	1.3	0.9	2.0	2.3	0.94	2.67	0.57
Lung ...	1.8	5.2	3.0	6.1	9.2	8.4	0.30	0.57	0.36
Spleen .	5.0	8.6	3.4	7.7	16.5	9.2	0.36	1.03	0.37
Thymus	3.8	5.3	4.1	26.4	27.6	16.2	0.14	0.19	0.25

question about the loss in heart DNA is very interesting and would deserve special consideration if a significant decrease had been established. As it is, we may take the fact that the rather large decrease is insignificant as an indication of the considerable variation existing in older animals. Microscopically, the myocardium was not remarkable in any way.

Besides DNA in the heart, only two other organs showed decreases in nucleic acids, viz., spleen and thymus. In the spleen W and TN remain essentially constant, whereas the nucleic acids are reduced to about half the maximum values recorded. In thymus not only the nucleic acids, but also W and TN are reduced, all quantities to about half the maximum value. This observation has also been made by ANDREASEN (1943) who determined total nucleic acid P in these organs. These changes are probably associated with the involution of the white pulp in the spleen (cf. HELLMAN 1926, HWANG, LIPPINCOTT and KRUMBHAAR 1938) and in the thymus (cf. HAMMAR 1940).

Changes in the protein-deficient animals.

Numerous studies have been undertaken to follow the changes occurring during protein deficiency in rats. There is not much of

this work in which the experimental conditions are quite comparable with those employed by us. As far as weight is concerned, our work is a repetition of the work by LIMSON and JACKSON (1932). These authors found it possible to divide organs into three groups with respect to the changes in weight when the growth of young rats was arrested by low-protein diet. The first group comprises organs showing significant increases, and of the organs investigated by us liver and heart are found in this group, the increases being about 16 and 5 per cent, respectively. Organs showing no change were included in the second group, in which we find kidney, brain and lung. In the third group, those organs showing significant decreases were included, and in this group spleen and thymus are listed, showing 48 and 64 per cent losses, respectively.

We did not find any increases for liver and heart, but otherwise our results agree well with those obtained by LIMSON and JACKSON. Whether or not this difference can be explained by the fact that we used only females, whereas LIMSON and JACKSON used half of each sex, cannot be said at the moment. The results of TN determinations are quite similar to the W curves, and no special discussion is therefore needed.

The influence of protein deficiency on the nucleic acid content of adult liver has been determined *e. g.* by CAMPBELL and KOSTERLITZ (1950), and by THOMSON et al. (1953), while MANDEL, JACOB and MANDEL (1950) have extended the investigations to other organs. The general conclusion from these investigations is that the number of nuclei (*i. e.* the amount of DNA) remains constant, even during very severe protein starvation, whereas considerable losses may occur for RNA. The only exceptions to this are brain, in which even RNA was found to remain constant, and spleen in in which DNA was found to decrease. Our experiments with growing rats deviate in certain respects.

As to DNA, this remains essentially constant in liver, kidney, and brain. In lung an indication of a slight increase is seen, but in heart there is a definite loss of DNA. Similarly, but less surprising, is the fact that DNA is lost in spleen and thymus. The findings for RNA are very similar to those for DNA. Thus, although a slight loss occurs in the liver during the first 10 days, this is apparently partly compensated later on. In the kidney RNA remains practically constant, while a slight loss probably occurs in the brain. In the lung RNA is higher, but not significantly so, after 4 months of protein deficiency, and in the heart a slight

loss of RNA is observed. In spleen and thymus were found losses parallel to the DNA losses.

The DNA constancy in the liver seems quite remarkable when compared with the histological observation that the number of nuclei increase. We found a ratio of about 2: 1 per square in starved and control animals (groups 4 and 1 respectively). Calculated on a volume basis this result indicates that there are about 3 times as many nuclei per unit volume in the experimental animals, and as the liver weight, and thus volume, is the same in both groups, the ratio of the total number of nuclei should also be 3. It is very difficult to explain this observation before further cytochemical studies have been carried out. One possibility is that polyploid liver cells divide during the period of protein deficiency, thus increasing the number of nuclei, without any change in the total DNA content. The results of FUKUDA and SHIBATANI (1953) show that in young rats (10 and 21 days) the amount of DNA per nucleus is rather close to that of normal diploid nuclei. In older male rats (41, 80, and 182 days) the amount of DNA per nucleus is about twice as high. Our results show a still larger effect from protein deprivation. The observed effect may, however, partly be explained by assuming that livers from starved animals contain more water, and thus lose more weight, and shrink more, during fixation. In connection with our results it may be mentioned that ELY and Ross have found during prolonged protein deficiency in young rats, that the amount of DNA per nucleus is increased, without being associated with any change in ploidy.

Our results confirm the remarkable constancy of DNA previously established, but it also emphasizes that during protein deficiency no net increase in DNA is possible. The exceptions are, as mentioned above, thymus and spleen, whose nucleic acid reserves are very labile, and heart. For this latter organ one is tempted to question whether this decrease in DNA is of a similar nature as that occurring in the normal heart during the last half of the experimental period, but as mentioned above, the decrease is not significant, and a discussion of the meaning of this finding must therefore be postponed. Our results seem to establish that in young rats the RNA is almost as "stable" metabolically as DNA. This would suggest that a larger part of the RNA is labile in adults than in the young. Other explanations are certainly imaginable, but no definite conclusions can be drawn.

Recovery in the protein-deficient animals.

The question about recovery of growth arrest caused by protein deficiency was investigated by JACKSON (1936). He found that subsequent to four months of protein deficiency it took about four months for females to reach the weight of the controls. When various organs were weighed, it was found that very few were significantly different in control and experimental animals. Among those organs investigated in the present work liver and spleen were significantly higher ($\sim$ 10 per cent) in the experimental animals (females).

Our results confirm this point completely, not only for W but for all quantities examined (except lung RNA), as no differences were observable after six months refeeding. This point is of some interest, especially from the point of view of DNA synthesis, *i. e.* increase in cell number. The question is whether cell divisions occurring during the normal growth process may be reversibly blocked for a shorter or longer period by protein deprivation. Superficially, our results would give an affirmative answer, so that the results of JACKSON could be extended to comprise not only a reversible retardation in organ weight gain but also a reversible retardation in the normal proliferation of cells.

Actually, the question may be raised whether this conclusion is correct. For such organs as heart and kidney whose regenerative power is known to be negligible, it is interesting if they undergo growth by cell division at an age when they normally are unable to regenerate by cell division. If this could be shown to hold, it would throw new light on the old question about the difference between growth and regeneration. Adaptive increase in size of the heart or kidney generally is considered not to be associated with increase in the number of cells. A glance at our curves will show that after the normal diet has been fed for two months DNA is still quite low in these two organs. It was in order to decide whether or not normal values were ever attained that the second batch of animals was used. As mentioned before, normal values were obtained in both organs, but in heart no change took place from the second to the eighth month. The answer is thus not decisive. As for the kidney we may ask whether the normal DNA content also corresponds to a normal histological structure, because admittedly DNA is quite a crude measure of the processes occurring

inside the organs. An increase in DNA could be accounted for by an infiltration of connective tissue cells.

A histological study of the kidney cortex did not show any increase in connective tissue in any group, control or experimental. Cell size of the tubular epithelium seemed to vary, however, those of fed and refed groups (3, 5, 6) being larger, those of group 1 controls and protein-deprived groups (2, 4) being smaller.

Summary.

1. Normal growing rats were fed a diet deficient in protein for varying periods up to four months. Data were collected on changes in organ weight, total nitrogen, DNA and RNA content per organ. Organs studied were liver, heart, kidney, brain, lung, spleen and thymus.

2. The constancy of the DNA content during protein starvation was confirmed. In the livers of protein-starved animals, while DNA per gram of tissue remained constant, histologically cell size diminished and the number of nuclei tripled. It is suggested that reduction of polyploid nuclei to the diploid form might partly account for this finding.

3. Alterations in RNA content paralleled those of DNA and seem to establish that in young rats RNA is almost as "stable" metabolically as DNA.

Supported by grants from the Swedish Cancer Society and the Swedish Medical Research Council.

We gratefully acknowledge the assistance of Mrs. JYTTE AHLMANN, cand. pharm., Miss INGER LAURENTIUSSON, Miss LILLEMOR NILSSON, Dr. J. KLINKEN-RASMUSSEN and Mr. BENGT WERDINIUS, med. kand.

References.

ANDREASEN, E., Acta path. microbiol. scand. 1943. Suppl. XLIX.
CAMPBELL, R. M. and H. W. KOSTERLITZ, J. Endocr. 1950. *6*. 308.
ELY, J. O. and M. H. ROSS, Science 1951. *114*. 70.
FITE, G. L., Ann. N. Y. Acad. Sci. 1954. *57*. 831.
FUKUDA, M. and A. SHIBATANI, J. Biochem. (Tokyo) 1953. *40*. 95.
HAMMAR, J. A., Z. mikr.- anat. Forsch. 1940. *49*. 68.
HELLMAN, T., Z. menschl. Vererb.- u. Konstit.lehre 1926. *12*. 270.
HWANG, J. M. S., S. W. LIPPINCOTT and E. B. KRUMBHAAR, Amer. J. Path. 1938. *14*. 809.

JACKSON, C. M., Amer. J. Anat. 1936. *58*. 179.
JACOB, M., L. MANDEL and P. MANDEL, Experientia. 1951. *7*. 269.
LESLIE, J., In E. CHARGAFF and J. N. DAVIDSON: The nucleic acids. N. Y. 1955.
LIMSON, M. and C. M. JACKSON, J. Nutr. 1932. *5*. 163.
MANDEL, P., M. JACOB and L. MANDEL, Bull. Soc. Chim. biol. (Paris) 1950. *32*. 80.
MASON, K. E., Amer. J. Physiol. 1940. *131*. 268.
SALKIND, J., Arch. Zool. 1915. *55*. 81.
THOMSON, R. Y., F. C. HEAGY, W. C. HUTCHISON and J. N. DAVIDSON, Biochem. J. 1953. *53*. 460.

Acta physiol. scand. 1958. 43. 71—76.

From the Department of Physiology, Faculty of Medicine (Karolinska Institutet), Stockholm 60.

The Effect of Acetaldehyde on the Secretion of Adrenaline and Noradrenaline from the Suprarenal Gland of the Cat.

By

E. S. PERMAN.

Received 7 March 1958.

Acetaldehyde is present in the blood in a concentration of 40—120 μg per 100 ml and is considered to be an intermediate in carbohydrate metabolism. The pharmacology of acetaldehyde was studied by HANDOVSKY (1934, 1936) who found that it produces a rise in the blood pressure, a stimulation of the respiration and a dilatation of the bronchial muscles when administered intravenously to anaesthetized dogs. A similarity between the blood pressure responses of acetaldehyde and adrenaline was noted by NELSON (1943) who also found that ligation of the suprarenals diminished the pressor action of acetaldehyde indicating an involvement of the medulla. NELSON apparently was the first to emphasize the sympathomimetic actions of acetaldehyde.

Interest in the pharmacology of acetaldehyde was renewed in 1948 mainly through the work of JACOBSEN and his associates. ASMUSSEN et al. (1948) showed that treatment with tetraethylthiuramdisulphide (Antabuse ®) sensitized human subjects to alcohol, causing a characteristic syndrome with flushing of the face, nausea, blood pressure fall, etc. Simultaneously with this reaction a large increase in the blood concentration of acetaldehyde was noted. Some of the symptoms of the so called Antabuse—alcohol reaction could be reproduced in human subjects by the intravenous infusion of acetaldehyde which therefore was thought to be chiefly

responsible for the reaction. The previously reported sympathomimetic effects could hardly explain its role in the Antabuse—alcohol reaction. Further studies of the pharmacodynamic actions of acetaldehyde have since been made by ROMANO, MEYERS and ANDERSON (1954). HERMANN, CHATONNET and VIAL (1955) studied the effects of acetaldehyde on various organs with sympathetic innervation and noted an increase in the pressor activity of the blood coming from the suprarenal after acetaldehyde administration. The effect was not abolished by splanchnic section. Their experiments were performed on cross-circulated dogs. No quantitative data on the suprarenal secretion were given.

The aim of the present study was to determine the amounts of adrenaline and noradrenaline released from the suprarenal medulla after acetaldehyde administration. The effect of acetaldehyde on the suprarenal might explain some pharmacologic actions of alcohol, as acetaldehyde is known to appear in the body during alcohol metabolism, and also give some new aspects on the Antabuse–alcohol reaction.

Material and Methods.

Cats were used in all experiments. They were not kept fasting and were anaesthetized with Nembutal 35 mg per kg body weight intraperitoneally. Heparin was given and blood from the left suprarenal vein was collected from a polyethylene tubing inserted into the corresponding lumbar vein, lateral branches being ligated. The arterial pressure was recorded from a carotid artery and was kept as constant as possible during the experiment by intravenous infusions of Ringer solution and plasma. This was done in order to avoid influences from variations in blood pressure on the secretion from the suprarenal medulla. In three experiments acute denervation of the suprarenals was done by cutting the splanchnic nerve on both sides just below the diaphragm. The acetaldehyde solution (4 per cent) was prepared in the cold (+ 4°C). It was given slowly intravenously after being warmed to body temperature in the syringe.

The suprarenal venous blood samples were centrifuged, the plasma poured off and stored at —30°C. Adrenaline and noradrenaline were determined by bioassay according to EULER (1949).

The experiments were usually started half an hour after the end of the operative procedure. First, samples of the "resting" secretion from the suprarenal were taken. Acetaldehyde was then given and further samples were collected at regular intervals. The experiments usually lasted about an hour. The hematocrit was read on each sample immediately after centrifugation. A drop of 5 per cent in the hematocrit value usually occurred during the course of the experiments.

Table I.

Amounts of adrenaline and noradrenaline release into the suprarenal vein after administration of acetaldehyde i. v. as compared to the resting secretion.

Exp. No.	Resting secretion mμg/min/kg		Dose of acetaldehyde	Secretion after acetaldehyde (7.5—50 mg/kg) mμg/min/kg	
	Adrenaline	Noradrenaline		Adrenaline	Noradrenaline
I. Innervated suprarenals	a. 5.2 b. 3.8	99 88	10 mg/kg	122	53
II. »	5.4	11.5	18.5 mg/kg	26.0	26.2
III. »	4.3	16.9	7.5 mg/kg	11.2	26.8
IV. »	4	27.4	11.7 mg/kg	16.2	45.5
I. Denervated suprarenals	20	42	10 mg/kg	94	15
II. »	<3	68	a. 25 mg/kg b. 50 mg/kg	10 23	56 39
III. »	<1	55	30 mg/kg	19	110

Results.

The resting secretion in 4 experiments ranged for adrenaline from 3.8 to 5.4 mμg/min/kg and for noradrenaline from 11.5 to 99 mμg/min/kg. After slow intravenous administration of acetaldehyde in doses from 7.5 to 18.5 mg/kg body weight an increase in the catechol amine content of the blood coming from the suprarenal was always noted (Table I). Blood drawn from the femoral vein at the same time was without effect on the bioassay systems used. The effect occurred during the first five minutes and seemed to have a short duration, the resting secretion being reattained within 20 minutes.

In three experiments acetaldehyde was given half an hour after acute section of the splanchnic nerves. The resting secretion was considerably lower in these experiments, especially for adrenaline which could not be demonstrated in the resting secretion in one experiment. Following acetaldehyde administration 10—50 mg/kg i. v. an increase in the secretion was found which was most

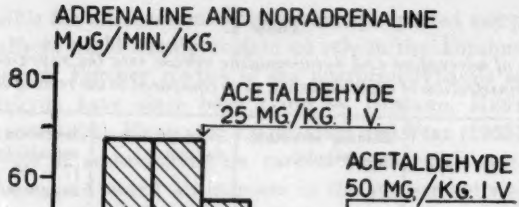


Fig. 1. Diagram of experiment no. 2 on denervated suprarenal.

0—10 min. Resting secretion.
10—25 min. Effect of acetaldehyde 25 mg per kg body weight i. v.
25—35 min. Effect of acetaldehyde 50 mg per kg body weight i. v.

pronounced for adrenaline (Table I). One experiment strongly indicates a differential secretion of adrenaline and noradrenaline (Fig. 1).

The administration of acetaldehyde seemed to have little effect on the blood pressure or on the respiration of the animals.

Discussion.

Indirect evidence that acetaldehyde brings about an increased secretion from the suprarenal medulla has earlier been presented. This investigation has shown that increased amounts of the medullary hormones appear in the suprarenal vein after administration

of acetaldehyde in doses which apparently have little effect on the circulation and respiration. The response is not abolished by acute section of the splanchnic nerves indicating that it is at least partly of peripheral origin.

An increase in the secretion from the adrenal medulla can be elicited by splanchnic stimulation, reflex liberation via the carotid sinus reflex (KAINDL and EULER 1951), hypoglycemia (DUNÉR 1954) and hypothalamic stimulation (BRÜCKE, KAINDL and MAYER 1952, FOLKOW and EULER 1954). A differential secretion of adrenaline and noradrenaline was observed in many of these experiments. Studying the secretion of the denervated suprarenal VOGT (1952) found no evidence for a differential secretion when potassium chloride was used to stimulate the chromaffin cells. The mechanism underlying the action of acetaldehyde, which seems to be a direct one, on the suprarenal medulla, is not known. The present results indicate a proportionally greater secretion of adrenaline than of noradrenaline but the mechanism for this has not been analyzed.

It is well known that acetaldehyde is present in the blood in increased amounts during the metabolism of alcohol. In a recent paper KLINGMAN and GOODALL (1957) report that the urinary excretion of adrenaline is high in dogs with severe alcohol intoxication. This state also causes a reduction of the adrenaline content of the suprarenal. Possibly this effect could be mediated by acetaldehyde formed during the breakdown of alcohol. In a similar way acetaldehyde could affect the suprarenal gland during the Antabuse—alcohol reaction.

Summary.

The secretion of adrenaline and noradrenaline from the suprarenal was determined by bioassay of suprarenal venous plasma collected from nembutalized cats. The administration of acetaldehyde 7.5—50 mg/kg regularly produced a marked increase in the secretion of adrenaline and noradrenaline. The increase was more pronounced for adrenaline. The effect remained after section of the splanchnic nerves. The results are discussed in relation to some pharmacological effects of alcohol.

This work was supported by a grant from Karolinska Institutet.

References.

Asmussen, E., J. Hald, E. Jacobsen, G. Jørgensen and V. Larsen, Acta pharmacol. (Kbh.) 1948. *4*. 285 — 332.

Brücke, F., F. Kaindl and H. Mayer, Arch. int. Pharmacodyn. 1952. *88*. 407.

Dunér, H., Acta. physiol. scand. 1954. *32*. 63.

Euler, U. S. v., Acta physiol. scand. 1949. *19*. 207.

Folkow, B. and U. S. v. Euler, Circulation res. 1954. *2*. 191.

Handovsky, H., C. r. Soc. Biol. (Paris.) 1934. *117*. 238.

Handovsky, H., C. r. Soc. Biol. (Paris.) 1936. *123*. 1242.

Hermann, H., J. Chatonnet and J. Vial, Arch. int. Pharmacodyn. 1955. *102*. 432.

Kaindl, F., and U. S. v. Euler, Am. J. Physiol. 1951. *166*. 284.

Klingman, G. I., and McC. Goodall, J. Pharmacol. 1957. *121*. 313.

Nelson, E. E., Proc. Soc. exp. Biol. (N. Y.) 1943. *52*. 23.

Romano, C., F. H. Meyers and H. H. Anderson, Arch. int. Pharmacodyn. 1954. *99*. 378.

Vogt, M., Brit. J. Pharmacol. 1952. *7*. 325.

Acta physiol. scand. 1958. 43. 77—81.

From the Department of Physiology, University of Turku, Turku, Finland.

Studies of the Effect of X-Rays on the Glucuronide Synthesis by the Gastric Mucous Membrane.

By

K. HARTIALA, V. NÄNTÖ and U. K. RINNE.

Received 18 March 1958.

It is known that ionizing radiation exerts an injurious effect on living cells and some of their functions. This is also true for X-rays which on the mucous membrane of the gastrointestinal tract cause degenerative changes. These changes may be reversible with moderate doses and irreversible with high doses. The degree of changes are also different for different types of cells. (IVY et al. 1923, IVY, MCCARTHY and ORNDOFF 1924, DAWSON 1925, MARTIN and ROGERS 1924 and BLOOM 1948).

Recent studies have shown that the gastrointestinal mucous membrane in rat also participates in the protective functions of the organism by means of carrying active detoxication glucuronide conjugations (HARTIALA 1954). In the present work we have studied the effect of irradiation on the detoxication capacity of the rat gastric mucosa.

Material and Methods.

Altogether 111 male rats (Wistar, weight 180—200 g) were used in the experiments. In order to protect the other parts of the animal from the X-rays the irradiation was performed so that the animal was covered with a 3 mm thick lead shield and only the ventricle was exposed through a mid-line excision and placed outside of

Table I.

The results expressed as μg of o-aminophenolglucuronide produced by the gastric mucous membrane per 100 mg dry weight tissue.

	Group A (400 r)			Group B (1,200 r)		
	Number of animals	μg/100 mg		Number of animals	μg/100 mg	
Controls	12	106±11.7		18	103±6.4	
Irradiated animals						
3 hours	13	75± 4.8	P<0.05	12	77±5.7	P<0.01
5 days	13	51± 5.5	P±0.001	14	45±1.9	P<0.001
12 days	7	73± 5.6	P=0.05	9	57±7.8	P<0.001
21 days after the radiation				6	62±5.2	P<0.01
Operated, but not irradiated animals (analysed 3h after operation	7	81± 5.2	P>0.05			

this shield. The operations were carried out under nembutal anesthesia and the exposed ventricle was kept moist with saline during the radiation period. After being irradiated, the stomach was replaced and the abdominal wall closed with silk sutures.

The detoxication synthesis was measured by using o-aminophenol as the toxic agent and analysing the amount of glucuronide formed from it. The analytic procedures have been described previously (HARTIALA and RONTU 1955). The experiments were made in two groups. In group A (33 experimental and 12 control animals) the rats received a 400 r (10 mA, 185 kV, 0.5 cmm Cu) radiation dose and the glucuronide synthesis capacity was determined 3 hours, 5 days, and 12 days after the X-ray application. Group B (41 test and 18 control animals) received 1,200 r (also 10 mA, 185 kV, 0.5 cmm Cu) and the analyses were carried out 3 hours, 5 days, and 21 days after the radiation treatment. Seven more rats were operated as in the previous groups and the corresponding glucuronide analyses were performed but without exposing the animals for radiation. These control experiments were done in order to find whether the operation itself exerted any effect as such.

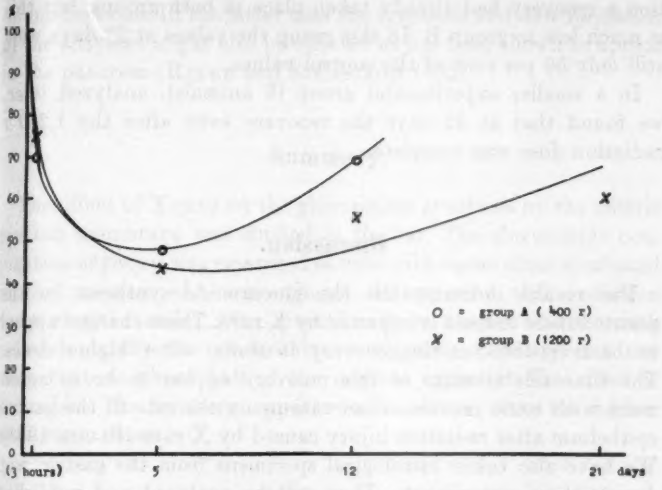


Fig. 1. Effect of X-ray-irradiation on the o-aminophenolglucuronide synthesis by the gastric mucous membrane. Results expressed as percentage changes from the simultaneous control values.

Results.

The results (expressed as μg of o-aminophenolglucuronide produced by the gastric mucous membrane per 100 mg dry weight tissue) are listed in the following table. In Fig. 1 the results are illustrated as percentage changes from the control values at different periods. It is evident that X-ray application in the given doses reduces the glucuronide detoxication capacity, but that these changes are still reversible. The immediate effects of the radiation procedure are seen already after 3 hours. At this time the conjugation capacity shows some 25 per cent reduction. Also the non-irradiated but operated animals showed a similar tendency for reduced values. The differences between the untreated control animals are not statistically significant. After 5 days the glucuronide synthesis was less than half of the control values. It appears that although these values in group B (receiving a higher X-ray dose) are slightly lower than with the smaller doses, the difference nevertheless is very small and lies within the methodical error. As to the late effects of irradiation the difference between the two groups becomes more prominent. At the 12th day after irradia-

tion a recovery had already taken place in both groups; but this is much less in group B. In this group the values at 21 days were still only 59 per cent of the control values.

In a smaller experimental group (5 animals), analyzed later, we found that at 32 days the recovery even after the 1,200 r radiation dose was complete.

Discussion.

The results indicate that the glucuronide synthesis by the gastric fundic mucosa is impaired by X-rays. These changes appear to be reversible but the recovery is slower after higher doses. The time relationships of this recovery appear to be in agreement with some previous observations on the rate in the gastric epithelium after radiation injury caused by X-rays (BLOOM 1948). We have also taken histological specimens from the gastric wall during these experiments. They will be analyzed and published later in connection of the β-glucuronidase enzyme-analyses, which were also performed in the same material. As is known the amount of this enzyme is claimed to parallel the amount of injury caused by different means (PELLEGRINO and VILLANI 1956, CERR, LEVRY and CAMBELL 1947, and CERR and LEVRY 1947).

As to the nature of the biochemical mechanism through which the reduction of the glucuronide synthesis by radiation is impaired very little can be said. The glucuronide synthesis itself, as has been shown in liver, comprises many reaction phases in which the energy rich phosphate compounds, as UTP and ATP, play an important role. Also the oxidation of the uridinediphosphoglucose to the corresponding UDP-glucuronide is a function of specific dehydrogenase enzymes (DUTTON and STOREY 1951, 1953, SMITH and MILLS 1954). At the moment no direct information is available concerning the effects of ionizing radiation on these particular systems. Our present studies now in progress are expected to elucidate some of these relationships. That enzyme systems are subject to the indirect action of radiation in aqueous solution (*e. g.* DALE 1940) as well as to direct action in the dry state (*e. g.* LEA 1944) is well known. The literature has recently been reviewed by ORD and STOCKEN (1953). The problem of the radiation effect on enzyme systems in the living cells becomes more complicated by the fact that the effect may be a direct one on the enzymes or

an indirect one. In the latter case the synthesis and new-formation of the enzymes might also be effected as has been shown to appear in the pancreas (RAUCH and STENSTROM 1952).

Summary.

The effect of X-rays on the glucuronide synthesis by the gastric mucous membrane was studied in the rat. The glucuronide conjugation capacity was measured *in vitro* with tissue slices incubated in the presence of o-aminophenol. It appeared that radiation doses of 400 r and 1,200 r given locally to the isolated stomach reduced equally the glucuronide conjugation capacity but not more than to about half of the original capacity. The effect is reversible. The recovery appears to be slower after the higher irradiation doses.

References.

BLOOM, W., Histopathology of Irradiation, McGraw-Hill Book Co Ltd New York. 1948.
CERR, L. M. H., G. A. LEVRY and J. G. CAMBELL, Nature 1947. *160*. 572.
CERR, L. M. H. and G. A. LEVRY, Nature 1947. *160*. 463.
DALE, W. M., Biochem. J. 1940. *34*. 1367.
DAWSON, A. P., Amer. J. Roentgenol. 1925. *13*. 320.
DUTTON, G. J. and I. D. E. STOREY, Biochem. J. 1953. *53*. 37.
DUTTON, G. J. and I. D. E. STOREY, Biochem. J. 1951. *48*. 29.
HARTIALA, K. J. W., Acta physiol. scand. 1954. *31*. Suppl. 114: 20.
HARTIALA, K. J. W. and S. RONTU, Ann. Med. exp. Biol. Fenn. 1955. *33*. 213.
IVY, A. C., J. E. MCCARTHY and B. H. ORNDOFF. J. Amer. med. Ass. 1924. *83*. 1977.
IVY, A. C., B. H. ORNDOFF, A. JACOBY and J. E. WITLOW, Radiology 1923. *1*. 39.
LEA, D., K. M. SMITH, B. HOLMES and R. MARKHAM, Parasitology 1944. *34*. 1367.
MARTIN, C. L. and F. T. ROGERS, Amer. J. Roentgenol. 1924. *11*. 280.
ORD, M. J. and L. A. STOCKEN, Physiol. Rev. 1953. *33*. 356.
PELLEGRINO, C. and G. VILLANI, Biochem. J. 1956. *62*. 235.
RAUCH, F. W. and K. W. STENSTROM, Gastroenterology 1952. *20*. 595.
SMITH, E. E. B. and G. T. MILLS, Biochim. biophys. Acta 1954. *13*. 386.

Acta physiol. scand. 1958. 43. 82—96.

From the Department of Histology, University of Lund, Lund, Sweden.

Isolation and Some Biochemical Properties of the Catechol Amine Granules in the Cow Adrenal Medulla.

By

NILS-ÅKE HILLARP.

Received 19 March 1958.

Previous studies have emphasized the necessity to get more exact information on the structure, composition and enzyme activities of the specific granules storing the catechol amines of the adrenal medulla. Without such information it does not seem possible to elucidate some essential problems concerning the storage and release of the amines. Several data of interest in this respect are, however, difficult to obtain unless specific granules of high purity are available. The present work is a first report of some experiments to separate the large granules of the adrenal medulla into fractions and to isolate the specific granules.

Material and Methods.

General procedure: By means of differential centrifugation the large granules of the cow adrenal medulla were separated into two main fractions. 1. A fraction of more easily sedimentable granules (G_w) which contains the larger part of the pressor amines. 2. A fraction (F_w) consisting of the remaining microscopic granules and particles which sediment as a fluffy layer on the top of the G_w fraction. The G_w granules were then separated into subfractions by density gradient centrifugation. The protein, pentose nucleic acid and amine contents and some enzyme activities were determined in the fractions.

Isolation of the medullary granules: For every experiment adrenal glands from 10 to 15 cows were used. The glands were removed from the body about 30 minutes after the killing of the animals and immediately cooled with ice. After 30 to 60 minutes such parts of the medulla that were free from cortical tissue were dissected out.

The medulla (15—20 g) was homogenized in 60 ml of 0.3 M sucrose as described in a previous paper (HILLARP, LAGERSTEDT and NILSON 1953). Unbroken cells and nuclei were removed by centrifugation at $800 \times g$ for 6 minutes. The supernatant was carefully decanted and diluted with 0.3 M sucrose to about 80 ml. It was centrifuged in two 50 ml tubes for 20 minutes at 15,000 r. p. m. (MSE superspeed 20′ refrigerated centrifuge; angle head 8 × 50 ml; $26,000 \times g$[1]). The supernatant was discarded and the looser layer above the more tightly packed bottom sediment was carefully removed by swirling with sucrose. The granules of the bottom sediment were washed twice with 40 ml of 0.3 M sucrose and each time the looser layer was removed. Finally the granules (G_w) were suspended in 0.3 M sucrose and made to a volume of 7.0 to 10.0 ml.

The granules of the looser top layer were diluted with sucrose, sedimented at 18,000 r. p. m. ($39,000 \times g$) for 20 minutes and washed once. The washed granules (F_w) were suspended in the same way as G_w. — All operations were performed at 0°.

Density gradient centrifugation: In each of eight 4 ml nylon tubes (fitting to the swing-out head of the MSE centrifuge) 1.0 ml of 1.5 M sucrose was layered above 2.0 ml of 1.8 M sucrose. Then 0.5 to 0.6 ml of the G_w suspension (or F_w suspension) was transferred to the top of the sucrose solution. After about 18 hours at + 4° C the tubes were centrifuged at 21,000 r. p. m. (about $50,000 \times g$) for 90 minutes. In this way a clear top layer (I in Fig. 1) and four subfractions of particles (II—V) were obtained.

In several of the experiments shown in the tables, the G_w suspension was not transferred to the tubes until one hour before the centrifugation took place. This resulted in a better recovery of amine granules in subfraction V (see Results).

Layers I to IV were removed by means of suction through a glass capillary with a bent tip.

The subfractions II to V were suspended in 0.015 M KCl at 0° C to get a lysis of the amine granules and thus a release of the catechol amines and the soluble proteins (cf. HILLARP 1958). They were then centrifuged for 45 minutes at 21,000 r. p. m. (angle head 8 × 10 ml; $38,000 \times g$) and washed once with 0.015 M KCl. The presence of an electrolyte resulted in an aggregation of the particles and thus facilitated the separation of the insoluble residue from the soluble proteins. In the clear supernatants no particles were found that could be spun down during one hour at $148,000 \times g$ (Spinco ultracentrifuge). The insoluble residue was suspended in water or 0.15 M KCl and then immediately used in the enzyme assays (and protein determinations). The KCl ex-

[1] All centrifugal forces are calculated for the bottom of the tubes.

tracts were used to determine the catechol amines and the soluble proteins of the subfractions.

In the experiments where the distribution of pentose nucleic acid was studied, the proteins of the subfractions were directly precipitated with trichloroacetic acid.

Aliquots of the G_w and F_w suspensions were in each experiment treated in the same way as the subfractions.

Enzyme assays: The enzyme activities of the insoluble residue obtained from G_w, F_w and the subfractions of G_w were determined.

The succinic dehydrogenase activity was assayed spectrophotometrically (reduction of cytochrome c) according to KUFF, HOGEBOOM and DALTON (1956). Control experiments showed that mitochondria from rat liver and cow adrenal cortex did not change their activity when treated in the same way as the medullary granules. Catechol amines in concentrations that maximally could have been present in the test system, did not influence the assays.

The ATPase activity was assayed by determining the inorganic phosphorus split off from ATP as described in a previous paper (HILLARP 1958). The incubation mixture contained 0.05 M histidine-HCl (ph 7.5), 0.04 M KCl, 0.005 M $MgCl_2$, 0.005 M ATP (ph 7.5), enzyme and water in a final volume of 3 ml. The ATPase activity of the G_w fraction is exclusively localized in the water insoluble residue and the activity is the same whether or not the water extractable contents of the granules are present (HILLARP 1958).

The adenylate kinase activity was assayed by determining the AMP formed when ADP was substrate in the above mixture. There is good evidence that the AMP formed in this system is produced by means of an ADP dismutation (HILLARP 1958). The incubation was carried out during 15 and 30 minutes at + 30° C. The enzyme amounts were adjusted to such a level that about one fourth of the ADP had undergone dismutation at the end of the incubations. The adenosinephosphates were determined by ion exchange chromatography as described in detail in previous papers (HILLARP 1958).

In all experiments suitable controls were used.

Other analyses: Adrenaline and noradrenaline were determined according to EULER and HAMBERG (1949), protein-N by the biuret method of CLELAND and SLATER (1953), phosphorous by the FISKE-SUBBAROW procedure (see LEPAGE 1951) and pentose nucleic acid according to SCHNEIDER (1945).

As G_w and F_w contain noradrenaline to about 30 per cent of the total pressor amines, an average molecular weight of 179 was used in the calculations of the total catechol amine content. This introduces only a small error when applicated to the subfractions.

Substances used: ATP (disodium salt) and ADP (monosodium salt) from the Pabst Laboratories or Sigma Chemical Company were neutralized to pH 7.5 with NaOH and kept at — 25° C. Cytochrome c (Sigma Chemical Company) was kept at — 25° C as a stock solution. The concentrations of ferri- and ferrocytochrome c were determined spectrophotometrically (cf. HOGEBOOM and SCHNEIDER 1952).

Results.

I. Catechol amines and proteins.

In the homogenates of cow adrenal medulla, usually about 80 per cent of the catechol amines were recovered in the granule fractions. The most easily sedimentable fraction (G_w) contained about 60 per cent of the amines bound to granules and had a considerably higher amine content per mg protein-N (24.3 μmoles; Table I) than the loosely packed top fraction (F_w) (12.5 μmoles).

By density gradient centrifugation the G_w granules were separated into four subfractions (II—V in Fig. 1). Just below the clear top layer (I) is a yellow-brown zone (II) which is distinctly delimited. Layer III has a white colour and is indistinctly delimited against the fourth zone where the granules gradually diminish in number. The bottom sediment (V) is tightly packed and has a weak pinkish colour.

The distribution of the catechol amines is recorded in Table I. In experiments 1 to 6 where the G_w suspension was transferred to the tubes at the same time as the sucrose solutions, a considerable amount of the amines was released from the granules and recovered in the clear top layer. This release is explained by the fact that many amine granules are damaged by osmotic lysis during the time period when the tubes are left standing to get a smooth sucrose gradient. No such extensive lysis was found when the G_w suspension was added one hour before the centrifugation took place (experiments 7 to 9). In this way a better recovery of amine granules was obtained in the bottom sediment as seen from the values for the distribution of catechol amines.

The main part of the catechol amines (79—84 per cent in experiments 7 to 9) was concentrated in layers III to V. Small amounts were also present in layer II but the amines may here at least partly originate from granules damaged by osmotic lysis and from a contamination by the top layer. The bottom sediment showed a very high and rather constant amine content per mg protein-N (34.7 μmoles). There are good reasons to believe that this sediment consists of almost pure specific granules (see Discussion). The subfractions III and IV had a considerably lower content (16.7 and 24.2 μmoles/mg N).

During the isolation procedure many of the specific granules are damaged in such a way that their amines and soluble proteins

Table I.

Catechol amines and soluble proteins in G_w and F_w and in the subfractions obtained at density gradient centrifugation of G_w. In experiments 7 to 9 the G_w suspension was not transferred to the gradient tubes until one hour before the centrifugation took place.

	Granule fraction	Experiment no.									Mean
		1	2	3	4	5	6	7	8	9	
Distribution of catechol amines in percent of G_w	I				38	35	36	13	10	11	
	II	6	6	6	6	6	8	8	5	6	
	III	14	11	13	16	16	16	19	19	17	
	IV				12	14		17	20	19	
	V	30	31	28	26	29	27	43	45	46	
Catechol amines µmoles/mg protein-N	II	8.30	8.70	9.50	12.5	13.0	14.0	14.5	8.60	10.0	11.0
	III	16.5	14.0	19.0	18.5	18.0	18.5	14.0	14.5	17.5	16.7
	IV				23.0	24.5		22.0	26.0	25.5	24.2
	V	34.5	36.5	37.0	36.5	34.5	33.0	30.5	34.0	35.5	34.7
	G_w		20.5	22.5	27.0	26.5	24.5	24.0	24.5	25.0	24.3
	F_w		9.15	10.5	15.0	13.5	13.0	14.5	13.0	11.5	12.5
Soluble proteins in per cent of total proteins	II		37	39	51	37	51	54			45
	III		64	76	76	69	74	66			71
	V		78	77	77	77	77	78			77
	G_w		61	67	66	59	70	68			65
	F_w		36	36	37	37	46	44			39

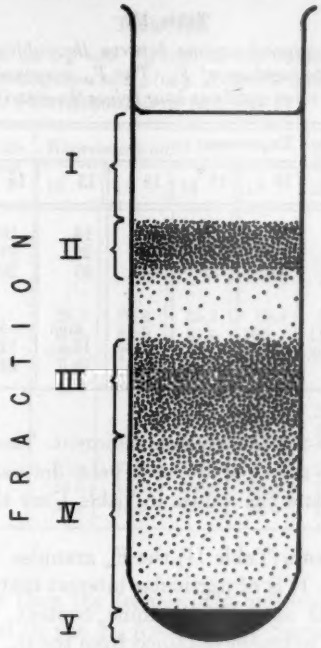


Fig. 1. Subfractions obtained at density gradient centrifugation.

are released (cf. HILLARP, LAGERSTEDT and NILSSON 1953, HILLARP and NILSSON 1954). The damaged granules sediment in the sucrose gradient to layer II and down into layer III. This could be shown in experiments where lytically damaged granules were centrifuged in gradient tubes. These subfractions therefore must be contaminated by damaged granules not containing any amines and soluble proteins, subfraction III probably to a relatively high extent.

The water soluble proteins represent the main part (77 per cent) of the proteins in the bottom sediment (Table I). This seems to be a rather unique feature of the specific granules. The proteins that may be extracted with a dilute KCl solution from mitochondria (isolated from rat and cow liver and cow adrenal cortex by the same method as for G_w), are, for instance, only 20—30 per cent of the total proteins. The value for subfraction III (71 per cent) is

Table II.

The distribution of catechol amines between the subfractions obtained at density gradient centrifugation of F_w. The F_w suspension was not transferred to the gradient tubes until one hour before the centrifugation took place.

	Granule fraction	Experiment no.						Mean
		10	11	12	13	14	15	
Distribution of catechol amines in per cent of F_w	II			17	14	10	12	
	III			34	26	27	32	
	V	30	32	31	35	29	31	
Catechol amines µmoles/mg protein-N	II			5.35	4.05	4.90	6.15	5.1
	III			10.5	12.5	12.0	11.5	11.6
	V	39.0	32.5	35.0	35.0	33.0	34.5	34.8

almost as high as for the bottom sediment. The soluble proteins of subfraction II may partly have been derived from damaged specific granules and the values in Table I are thus probably too high.

In six experiments (Table II) the F_w granules were fractionated in gradient tubes. It is of particular interest that the bottom sediment showed the same high amine content (34.8 µmoles/mg protein-N) as the sediment obtained from the G_w fraction. Another point of interest is that about the same amount of catechol amines were recovered in subfractions III and V.

The adrenal medulla of various animals has two different types of cells, one type which stores mainly adrenaline and one mainly noradrenaline (HILLARP and HÖKFELT 1953—55, ERÄNKÖ 1955). Because of this there must exist specific granules of two different types containing mainly or exclusively adrenaline and noradrenaline respectively. In some of the experiments the distribution of both amines was determined. Confirming the results obtained by EADE (1956) in a similar study, it was found that the specific granule subfraction of lower density (III) has a considerably higher adrenaline content (in per cent of the total amines) than the subfraction of high density (V). It may thus be possible to separate the two granule types from each other.

II. Pentose nucleic acid.

It is obvious from the procedure used for isolation of G_w and F_w that the latter fraction must be contaminated by microsomes

Table III.

Pentose nucleic acid in G_w and F_w and in the subfractions obtained at density gradient centrifugation of G_w (exp. 16 to 18) and F_w (exp. 19 to 22). Centrifugations as in Table II.

PNA-pentose expressed as µg ribose	Granule fraction	Experiment no.						
		16	17	18	19	20	21	22
Total	I						3.3	2.1
	II				118	100	76.0	92.0
	III				82.0	78.0	42.0	51.5
	IV						20.5	23.0
	V	26.5	20.0	25.5	19.0	36.0	16.5	17.0
	G_w	68.0	53.0	61.0	105	117	60.0	67.5
	F_w	163	118	132	262	287	165	194
Per mg protein-N	I						9.9	7.1
	II				59.0	33.5	51.5	48.0
	III				30.5	20.5	27.0	26.5
	IV						30.0	27.5
	V	25.5	21.5	22.0	20.5	25.0	27.0	24.0
	G_w	20.0	17.5	18.0	22.5	19.0	19.0	21.5
	F_w	30.5	24.5	25.5	35.0	27.0	33.0	32.0

to a higher degree than the former. The mitochondrial material is also concentrated in the F_w fraction (see Section III). It may thus be expected (cf. KUFF and SCHNEIDER 1954, PALADE and SIEKEVITZ 1956) that F_w should have the highest content of pentose nucleic acid (PNA). In good agreement with this, Table III shows that the main part (71 per cent of the PNA-pentose was found in F_w.

At density gradient centrifugation of F_w (experiments 19 to 22 in Table III) the main part (about 70 per cent) of the PNA-pentose was recovered in subfractions II and III. Subfraction II also showed the highest content per mg protein-N. Only about 10 per cent was found in the bottom sediment. When G_w was fractionated (experiments 16 to 18) about four times more PNA-pentose was recovered in the sediment. In spite of this, however, the content per mg protein-N was practically the same in the bottom sediment from G_w as that from F_w. It is also of special interest that the absolute amounts of PNA-pentose in the sediments from F_w and G_w were about the same in spite of the fact that the total amounts in the starting material were several times larger when the F_w suspensions were fractionated.

Table IV.

Succinic dehydrogenase activity of G_w, F_w and subfractions of G_w. Activity is expressed in micromoles of cytochrome c reduced per minute (specific activity: per mg of protein-N.

Succinic dehydrogenase activity	Granule fraction	Experiment no.			
		23	24	25	26
Total activity	II	0.467	0.336	0.350	0.216
	III	0.092	0.110	0.034	0.057
	V	0.012	0.015	0.009	0.012
	G_w	0.615	0.484	0.416	0.320
	F_w	2.35	1.87	2.68	2.84
Specific activity	II	1.27	0.840	0.980	0.695
	III	0.288	0.281	0.116	0.218
	V	0.047	0.054	0.035	0.032
	G_w	0.305	0.216	0.290	0.211
	F_w	0.525	0.396	0.560	0.600
Total activity in per cent of G_w	II	76	70	84	68
	III	15	23	8.2	18
	V	1.9	3.1	2.2	3.7

III. Succinic dehydrogenase.

As the medullary granules are isolated in a similar way as mitochondria, it is necessary to know if and to what extent the granules are contaminated by these particles. The occurrence of the typical mitochondrial enzyme, succinic dehydrogenase, was therefore studied. This enzyme was chosen because of its high stability and because its activity may be easily determined even when only small amounts of enzyme are available. Another advantage is that the enzyme is not solubilized by extraction of the mitochondria with water. When catechol amines are present, such an extraction is necessary in order to remove the amines which are readily oxidized by cytochrome c (cf. FALK 1949).

Both the total and the specific activity of the succinic dehydrogenase was considerably higher in the F_w than in the G_w fraction (Table IV). This is in total agreement with the fact that the amine granules have a considerably higher density than the mitochondria (see below). The low total activity of G_w as compared to that of F_w indicates that most of the mitochondria of the homogenates are removed from the specific granules if the G_w fraction is isolated by the technique used in the present work.

Table V.

ATPase activity of G_w, F_w and subfractions of G_w. Activity expressed in micromoles of inorganic P liberated per hour (specific activity: per mg of protein-N).

ATPase activity	Granule fraction	Experiment no.			Mean
		27	28	29	
Total activity	II	13.9	27.8	19.8	
	III	19.9	33.0	21.1	
	V	27.5	29.0	26.4	
	G_w	106	141	98	
	F_w	226	299	161	
Specific activity	II	24.0	26.0	24.5	24.8
	III	56.9	47.9	60.2	55.0
	V	64.0	66.0	67.6	65.9
	G_w	51.1	45.5	42.4	46.3
	F_w	53.5	52.0	49.8	51.9
Total activity in per cent of G_w	II	13	20	20	18
	III	19	23	22	21
	V	26	21	27	25

The distribution of the enzyme activity between the subfractions II, III and V obtained by density gradient centrifugation is shown in Table IV. The recovery of the total activity was good (93—96 per cent) in three of the experiments and only a little lower (89 per cent) in one of them. The loss is no doubt explained by the fact that the zone between II and III was discarded. It is clearly seen from the distribution of total activity that the mitochondria were mainly accumulated in layer II in which 68—84 per cent of the total G_w activity was located. The mitochondria sedimented also to a minor extent down to layer III. The distribution of total activity as well as the specific activities of the subfractions show that the specific granules in III were to a small extent contaminated by mitochondria. The amine granules in the bottom sediment, on the other hand, were practically free from mitochondria. The very small total activity found in this subfraction may well be due to an experimental error. This is in good agreement with observations made in other experiments. Liver mitochondria (rat and cow) were fractionated in the same way as the G_w suspension. Most of them were recovered in a layer corresponding to II in Fig. 1 and some sedimented down to a position corresponding to III but no mitochondria were forced down to the bottom of the tubes. The results obtained by density gradient

centrifugation of mitochondria (HOLTER, OTTESEN and WEBER 1953, OTTESEN and WEBER 1955, KUFF and SCHNEIDER 1954) performed with a technique similar to or different from the one used in the present work also clearly show that mitochondria have a density that is considerably lower than the density of the specific granules of subfraction V.

IV. ATPase and adenylate kinase.

The G_w fraction shows a high ATPase activity bound to the water insoluble proteins (HILLARP 1958). The F_w and G_w fractions had about the same specific activity but the total activity of F_w was about twice as high as that of G_w (Table V).

The recovery of the ATPase activity in subfractions II, III and V was only about 64 per cent. This is in all probability due in a large part to the loss of the granules present in the zone between III and V. Only about 18 per cent of the total ATPase activity of G_w was recovered in subfraction II which, however, contains about three fourths of the succinic dehydrogenase activity. Besides this, a part of the ATPase activity in subfraction II must belong to damaged specific granules. Although mitochondria have a high ATPase activity (KIELLEY and KIELLEY 1951, 1953, NOVIKOFF et al. 1952, LARDY and WELLMAN 1953[1], the above data clearly show that the ATPase activity of the G_w fraction only to a minor degree can be derived from mitochondria. Unless the mitochondria of the adrenal medulla have an unusually low specific activity, this gives a further support to the view that the contamination of the G_w fraction by mitochondria is small.

Subfraction V had the highest specific ATPase activity and there is no doubt (see Discussion) that the activity is located in the amine granules. Only a somewhat lower specific activity was found in subfraction III.

The G_w fraction contains one adenylate kinase that is bound to the insoluble residue obtained by extraction with a dilute KCl solution and another adenylate kinase that is released from the granules during the extraction procedure (HILLARP 1958). The bound ADP transphosphorylase is not removed by repeated washings and is heat-stable in acid.

[1] Rat liver mitochondria, isolated in the same way as the G_w fraction, showed a specific activity of about 90 μmoles P/mg N/hour when tested in the same assay system as used for the medullary granules. Cow liver mitochondria were found to have a considerably lower activity, however.

It has as yet not been possible to get reliable data concerning the distribution of the adenylate kinase activity between the subfractions. A factor complicating the assays is that it is not known to what extent the procedures for the isolation of the soluble proteins and for the heat inactivation of the ATPase changes the adenylate kinase activity. There are furthermore technical difficulties involved in testing the small fractions obtained by the density gradient centrifugation. For the above reasons only subfraction V has been studied.

In similarity to G_w, the subfraction V contained both a bound and a soluble ADP transphosphorylase. The specific activities (bound enzyme: 20—30 μmoles ADP dismutated/mg N/hour; soluble enzyme: 10—15 μmoles) are considerably lower than the activities of rat liver mitochondria assayed in a test system similar to that used in this work (SIEKEVITZ and POTTER 1953, SIEKEVITZ and WATSON 1956). It is rather possible, however, that the bound enzyme had been more or less inactivated during the heating in acid. For unknown reasons the insoluble residue showed no activity in some experiments.

Discussion.

The density gradient centrifugations show that a large part of the specific amine granules in the cow adrenal medulla has a high density and sedimentation rate in hypertonic sucrose solutions. The mitochondria in the medullary cells have a considerably lower density (or possibly sedimentation rate). It may thus be possible to separate these two types of granules from each other and the experiments make it highly probable that the specific granules obtained in subfraction V are free from mitochondria. Other cell particles may have a density as high as or higher than the amine granules. Microsomal material from liver cells has, at least partly, a high density (HOLTER, OTTESEN and WEBER 1953, KUFF and SCHNEIDER 1954, OTTESEN and WEBER 1955). The microsomes, however, have a low sedimentation rate owing to their small size and the gradient centrifugations in the present work were performed with use of a relatively weak centrifugal force and in a relatively short time. It seems therefore hardly probable that the specific granules in the bottom sediment contain microsomal material as contaminant. Good support for this view is obtained from the results in the experiments where the

F_w granules were fractionated in gradient tubes. In spite of the fact that there must be much larger amounts of microsomes in F_w than in G_w, subfraction V isolated from F_w had the same high content of catechol amines and the same low content of PNA-pentose per mg protein-N as the bottom sediment obtained from G_w. Furthermore, the absolute amounts of PNA-pentose in the sediments from F_w and G_w were about the same in spite of the fact that the total amounts in the starting material were several times larger when the F_w granules were fractionated. These results indicate that the low sedimentation rate of the microsomal material prevents them from sedimenting down into subfraction V.

It may be concluded that the sediment obtained at density gradient centrifugation of G_w is free or practically free from mitochondria and microsomes. The specific granules in this sediment may be contaminated by particles of the large granule sort other than mitochondria. No such particles are found in the chromaffin cells (WETZSTEIN 1957), however, and their number — if originating from other cells in the medulla — must be exceedingly small as compared to the number of specific granules.

The G_w fraction displays a high ATPase activity which only to a minor degree can be derived from mitochondria. The distribution of the activity as well as the specific activity found in the subfractions clearly show that the larger part of the ATPase activity is localized in the amine granules. This is of special interest in view of the fact that the specific granules store large amounts of adenosinetriphosphate together with the catechol amines (HILLARP, HÖGBERG and NILSON 1955, HILLARP 1958).

The specific granules with high density have a very high catechol amine content. The amine granules with lower density (or sedimentation rate) seem to have a considerably lower content as there are no indications that they are heavily contaminated by other particles. On the contrary, such a contamination is contradicted by the observations that subfraction III has a low succinic dehydrogenase and high ATPase activity and shows almost the same very high value for soluble proteins as subfraction V. It cannot be concluded, however, that there are two types of amine granules differing in amine content as the appearance of layer III may have been caused by the non-linearity of the density gradient.

In a previous work it was not possible to demonstrate any mitochondria in the cow adrenal medulla by use of aniline acid fuchsin staining according to ALTMANN (HILLARP, HÖKFELT and

NILSON 1954). It was therefore assumed that the succinic dehydrogenase activity found in the fraction of specific granules was localized in these granules. The results in the present work show, however, that this assumption was erroneous. This is in good agreement with the results obtained by BLASCHKO et al. (1957) in experiments where particles with fumarase activity could be separated from the amine granules in bovine adrenal medulla by use of density gradient centrifugation.

Summary.

1. By means of density gradient centrifugation the catechol amine granules in the cow adrenal medulla have been isolated free or practically free from mitochondria and microsomal material.

2. The isolated granules have a very high content of pressor amines (about 35 μmoles/mg protein-N). The soluble protein fraction that is released together with the amines at osmotic lysis of the granules, represents the main part (77 per cent) of the total granule proteins.

3. The granules show a high ATPase activity and probably contain an adenylate kinase.

This work was aided by a grant from the National Medical Research Council of Sweden.

References.

BLASCHKO, H., P. HAGEN, J. M. HAGEN and H. J. SCHÜMANN, Arch. int. Pharmacodyn. 1957. *110*. 128.

CLELAND, K. W. and E. C. SLATER, Biochem. J. 1953. *53*. 547.

EADE, N. R., J. Physiol. 1956. *132*. 53. P.

ERÄNKÖ, O., Nature. 1955. *175*. 88.

EULER, U. S. v. and U. HAMBERG, Acta physiol. scand. 1949. *19*. 74.

FALK, J. E., Biochem. J. 1949. *44*. 369.

HILLARP, N.-Å., Acta physiol. scand. 1958. *42*. 321.

HILLARP, N.-Å., B. HÖGBERG and B. NILSON, Nature. 1955. *176*. 1032.

HILLARP, N.-Å. and B. HÖKFELT, Acta physiol. scand. 1953. *30*. 55.

HILLARP, N.-Å. and B. HÖKFELT, Endocrinology 1954. *55*. 255.

HILLARP, N.-Å. and B. HÖKFELT, J. Histochem. and Cytochem. 1955. *3*. 1.

HILLARP, N.-Å., B. HÖKFELT and B. NILSON, Acta anat. (Basel) 1954. *21*. 155.

HILLARP, N.-Å., S. LAGERSTEDT and B. NILSON, Acta physiol. scand. 1953. *29*. 251.

HILLARP, N.-Å. and B. NILSON, Acta physiol. scand. 1954. *32*. 11.
HOGEBOOM, G. H. and W. C. SCHNEIDER, J. biol. Chem. 1952. *194*. 513.
HOLTER, H., M. OTTESEN and R. WEBER, Experientia 1953. *9*. 346.
KIELLEY, W. W. and R. K. KIELLEY, J. biol. Chem. 1951. *191*. 485.
KIELLEY, W. W. and R. K. KIELLEY, J. biol. Chem. 1953. *200*. 213.
KUFF, E. L., G. H. HOGEBOOM and A. J. DALTON, J. Biophys. and Biohem. Cytol. 1956. *2*. 33.
KUFF, E. L. and W. C. SCHNEIDER, J. biol. Chem. 1954. *206*. 677.
LARDY, H. A. and H. WELLMAN, J. biol. Chem. 1953. *201*. 357.
LEPAGE, G. A., in W. W. UMBREIT, R. H. BURRIS and J. F. STAUFFER, Manometric Techniques, 2nd dit. p. 185. Minneapolis 1951.
NOVIKOFF, A. B., L. HECHT, E. PODBER and J. RYAN, J. biol. Chem. 1952. *194*. 153.
OTTESEN, M. and R. WEBER, C. R. des travaux du Labor. Carlsberg Série Chimique. 1955. *29*. 417.
PALADE, G. E. and P. SIEKEVITZ, J. Biophys. and Biochem. Cytol. 1956. *25*. 171 and 671.
SCHNEIDER, W. C., J. biol. Chem. 1945. *161*. 293.
SIEKEVITZ, P. and R. V. POTTER. J. biol. Chem. 1953. *200*. 187.
SIEKEVITZ, P. and M. WATSON, J. Biophys. and Biochem. Cytol. 1956. *2*, 653.
WETZSTEIN, R., Zeitschr. Zellforsch. 1957. *46*. 517.